Expression of Functional M$_2$ Muscarinic Acetylcholine Receptor in Escherichia coli$^1$

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The M$_2$ muscarinic acetylcholine receptor mutant (M$_2$ mutant), with a lack of glycosylation sites, a deletion in the central part of the third inner loop, and the addition of a six histidine tag at the C-terminus, was fused to maltose binding protein (MBP) at its N-terminus and expressed in Escherichia coli. The expression level was 0.2 nmol receptor per 100 ml culture, as assessed as [3H]-quinuclidinyl benzilate ([3H]QNB) binding activity, when the BL 21 strain was cultured at 37°C to a late growth phase and the expression was induced by isopropyl β-thiogalactoside at 20°C. No [3H]QNB binding activity was detected when it was not fused to MBP or when expression was induced at 37°C instead of 20°C. The MBP-M$_2$ mutant expressed in E. coli showed the same ligand binding activity as the M$_2$ mutant expressed in the Sporodoptera frugiperda (Sf9)/baculovirus system, as assessed as displacement of [3H]QNB with carbachol and atropine. The MBP-M$_2$ mutant was solubilized, purified with Co$_{2+}$-immobilized Chelating Sepharose gel and SP-Sepharose, and then reconstituted into lipid vesicles with G protein G$_{i/o}$ in the presence or absence of cholesterol. The reconstituted vesicles showed GTP-sensitive high affinity binding for carbachol. Carbachol and carbachol-stimulated [3H]GTPyS binding activity was the same as those observed for the M$_2$ mutant expressed in Sf9 cells and were not affected by the presence or absence of cholesterol. These results indicate that the MBP-M$_2$ mutant expressed in E. coli has the same ability to interact with and activate G proteins as the M$_2$ mutant expressed in Sf9, and that cholesterol is not essential for the function of the M$_2$ muscarinic receptor.

Key words: cholesterol, Escherichia coli, G protein–coupled receptor, muscarinic acetylcholine receptor, reconstitution.

The muscarinic acetylcholine receptor belongs to this superfamily of GPCRs. There are five subtypes of muscarinic receptors (1, 2): the M$_1$, M$_3$, and M$_4$ subtypes couple to G$_{i/o}$ type G proteins, and the M$_2$ and M$_5$ subtypes couple to G$_{q}$ type G proteins. Muscarinic receptors are broadly expressed from peripheral tissues such as salivary glands (M$_1$ and M$_3$), sweat glands (M$_3$), ileum (M$_2$, M$_3$), and heart (M$_2$), to the central nervous system (M$_1$–M$_5$).

The expression levels of GPCRs are usually less than 1 pmol/mg protein. The development of a high expression system is essential for molecular characterization of GPCRs including muscarinic receptors, particularly for structural studies involving electron or X ray crystallography. The system involving baculovirus and insect cultured cells like Sf9 has been successfully used for large scale expression of GPCRs (5–8). Muscarinic receptors have been expressed in this system, and have been shown to have the same ligand binding and G protein activating activity as those extracted from mammalian tissues or expressed in mammalian cultured cells (5, 9, 10). However, considering the time and cost required for culturing Sf9 cells, a more easily handled expression system is desirable. Muscarinic receptors have also been expressed in Saccharomyces cerevisiae (11) and BY 2 tobacco cells (12), although the expres-

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Abbreviations: M$_2$, M$_2$ muscarinic acetylcholine receptor; G protein, guanine nucleotide–binding regulatory protein; GPCR, G protein–coupled receptor; MBP, maltose binding protein; E. coli, Escherichia coli; Sf9, Sporodoptera frugiperda; S-IT, 5-hydroxytryptamine (serotonin); IPTG, isopropyl β-thiogalactoside; [3H]QNB, [3H]-quinuclidinyl benzilate; [3H]NMES, [3H]-methylisocapamine; GTPyS, guanosine 5′(3′-O-thiotriphosphate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; DTT, dithiothreitol; GST, glutathione S-transferase; Trx, thioredoxin.

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sion levels in both systems were low: 20 fmol/mg protein for *S. cerevisiae* and 2.0-2.5 pmol/mg protein for BY 2 tobacco cells. Because of its ease in culturing, *E. coli* is considered to be one of the most efficient expression systems for acquiring proteins of interest. There have been several reports on the expression of GPCRs in *E. coli* (13). The β-adrenergic receptor was first expressed among GPCRs and was found to retain ligand binding activity (14). The 5HT1A receptor expressed in *E. coli* was reported to interact with G proteins (15). However, the expression levels in these studies were low. Grishhammer et al. have shown that a relatively high amount of neutrotensin receptor can be expressed in *E. coli* through the fusion of MBP at the N-terminus of the receptor (15 pmol/mg protein) (16, 17). The M₁ muscarinic receptor (18) and dopamine D₂ receptor (Ito and Haga, unpublished data) have also been expressed successfully using the same strategy as for the neutrotensin receptor. These receptors have been shown to have the same ligand binding activity as mammalian receptors, but it remains unknown if they have the same ability to interact with G proteins. In the present study, we have expressed the M₄ muscarinic receptor as a MBP fusion protein in *E. coli* using the strategy used for the expression of neutrotensin receptors (16, 17). The expressed receptor, which was purified and reconstituted into lipid vesicles, was found to have the same ligand binding and G protein activating activities as the M₂ muscarinic receptor expressed in Sf9 cells.

We have also examined if cholesterol is necessary for the function of muscarinic receptors. Cholesterol, either by changing the physical properties of biological membranes or by directly interacting with proteins, can modulate the function of membrane proteins (19). A well known example is the nicotinic acetylcholine receptor, which loses its cation channel function in the absence of cholesterol (20, 21). There have been several reports on the role of cholesterol in the function of GPCRs; for some receptors including β-adrenergic (22), muscarinic (23), and oxytosin receptors (24-26), cholesterol has been reported to be required for retention of ligand binding activity or for increasing the affinity of receptors for ligands, and for other receptors including dopamine D₂ receptor (27) and rhodopsin (28), cholesterol has been reported to inhibit their activation. These results were obtained through experiments involving membrane preparations or proteoliposomes in which detergent-solubilized proteins were reconstituted, and these preparations may contain various levels of cholesterol. Definite answers regarding the requirement of cholesterol should be obtained through experiments involving the reconstitution of purified and cholesterol-free GPCRs and G proteins in a defined composition of lipids. We took advantage of the fact that the membrane of *E. coli* does not contain any cholesterol (29). Here, we provide evidence that the muscarinic receptor can activate G proteins in the absence of cholesterol.

**MATERIALS AND METHODS**

**Materials**—PET25b(+), BL21, and BL21(DE3) were purchased from Novagen; pGEX-2T, Chelating Sepharose fast flow, SP-Sepharose, and Sephadex G50 from Pharmacia; GTP, GTPγS, GDP, PC, PI, cholesteryl hemisuccinate, CHAPS, and digitonin from Sigma; sodium cholate from WAKO; [PH]NMS, [PH]QNB, and [35S]GTPγS from NEN Dupont; His-probe and Goat anti rabbit IgG (horseradish peroxidase) from Santa Cruz; PVDF membranes from Milipore; and GF/B glass fiber filters from Whatman. The pRGI/MBP and pT/Trx vectors were kind gifts from Dr. Grishhammer (MRC Center, Cambridge, UK) and Dr. Ishii (RIKEN, Tsukuba), respectively.

**Strains, Expression Vectors, and Culture Conditions**—The expression vector pRGI/MBP was used in the present experiment. This expression vector contained most of MBP under the control of the lac promoter and the double ribosomal binding site of the vector pASK40 (17). We used the M₄ muscarinic receptor mutant (M₄ mutant), M₄(N-D) (6His)(3del) (10), in which most of the third internal loop (233-330) was deleted, a six histidine tag (6xHis) and a thrombin cleavage site were added to the C-terminus, and Asn 2, 3, 6, and 9 were replaced by Asp to prevent N-glycosylation (Fig. 1a) (10). Various expression vectors were constructed to express the M₄ mutant (Fig. 1b).

The M₄ mutant starting from Asp 2 was fused to MBP at its N-terminus between the *BamHI* and *PstI* sites of the pRGI/MBP vector (pRGI/MBP-M₄ mutant). The same coding sequence as that of the pRGI/MBP-M₄ mutant was inserted down stream of the T7 promoter and a ribosomal binding site of PET25b(+) between the NcoI and HindIII sites. The resultant vector, the PET25(+)-MBP-M₄ mutant, contained a pel B signal sequence (30) at its N-terminus. The PET25(+)-MBP-M₄ mutant was digested with NdeI and NcoI, followed by blunt end and ligation to remove sequences that encode pel B to construct the PET25(-)MBP-M₄ mutant. The PET25(+)-MBP-M₄ mutant was constructed by inserting the whole sequence of the M₄ mutant between the NcoI and HindIII sites of PET25b(+). The thioredoxin (Trx) sequence derived from *E. coli* strain K12 was isolated from the vector pT/Trx, which includes Trx under the control of the T7 promoter (31), by PCR. The resultant Trx gene was inserted into the C-terminus of the MBP-M₄ mutant between the NcoI and HindIII sites of PET25b(+) (32). The trimeric thioredoxin (Trx) sequence was derived from *E. coli* strain K12. The pRGI/MBP-M₄ mutant-Trx(-His) did not include 6xHis between the M₄ mutant and Trx. GST was also inserted into the N-terminus of the M₄ mutant. The pGEX-2T vector was digested with *BamHI* and EcoRI, and then blunted-ended. The whole sequence of the M₄ mutant (between NcoI and PstI) was blunt-ended and then inserted into the vector (pGEX-M₄ mutant).

The pRGI/MBP and pGEX based vectors were transformed into BL21, and the PET25(+)-MBP-M₄ mutant and PET25b(+) based vectors were transformed into BL21(DE3) and selected for ampicillin resistance. Also, for expression of thioredoxin, both the pRGI/MBP-M₄ mutant and pT/Trx were transformed into BL21(DE3) and selected for both ampicillin and chloramphenicol resistance. BL21 was grown in 2xTY medium containing 100 mg/ml ampicillin for both ampicillin and chloramphenicol resistance. BL21 was grown in 2xTY medium containing 50 mg/ml ampicillin 24 h later and stored at -80°C. The M₄ mutant was expressed in Sf9/baculovirus, in the same manner as previously described (5, 10).

**Membrane Preparation**—Spheroplasts were obtained by the method as described previously (32, 33). The cell pellet derived from 1 liter culture was resuspended in 100 ml of a
Expression of Muscarinic Receptor in E. coli

Fig. 1. Topology of the M2 mutant and the expression constructs. (a) The mutations of the M2 muscarinic receptor included deglycosilation at the N-terminal domain, deletion of the central part of the third inner loop, and the addition of a thrombin cleavage site and a 6xhistidine tag. (b) Various fusion receptors with various promoters were obtained for expression.

buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 25% sucrose, 0.5 mM phenylmethylsulphonyl fluoride, and 60 μg/ml lysozyme, and stirred at 4°C for 1 h. An equal amount of cold deionized water was added and then mild sonication was performed [30 s on and 2 min off at level 5 for three times, with a Tomy ultrasonic disrupter (UD-200)]. The membrane fraction was collected by centrifugation at 150,000 xg at 4°C for 1 h. The pellet was resuspended in 100 ml of a buffer containing 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 1 mM EDTA, and then centrifuged at 150,000 xg at 4°C for 1 h. The pellet obtained on the last centrifugation was resuspended in 25 ml of 20 mM Tris-HCl (pH 7.4).

Ligand Binding Assay—The membrane fraction was assayed for its ability to bind to muscarinic antagonists, [3H]QNB or [3H]N-methylscopolamine ([3H]NMS). The method for the binding assay was described previously (34). The membrane (20 μl from the above suspension) was incubated in 1 ml of 20 mM potassium phosphate buffer (KPB) (pH 7.0) containing [3H]QNB or [3H]NMS at 30°C for 1 h. [3H]QNB or [3H]NMS bound to receptors was trapped on a GF/B glass fiber filter and then the radioactivity was measured with a liquid scintillation counter.

The membrane fraction was solubilized with 1% digitonin and 0.3% sodium cholate at 2 mg/ml protein concentration. The solubilized receptor (10 μl) was incubated in 200 μl of a buffer containing 20 mM potassium phosphate buffer (pH 7.0), 100 mM NaCl, 0.1% digitonin and [3H]QNB or [3H]NMS at 30°C for 1 h with different concentrations of carbamylcholine and atropine. [3H]QNB or [3H]NMS bound
to solubilized receptors was recovered in the void volume fraction from a Sephadex G50 column and then the radioactivity was measured with a liquid scintillation counter.

Western Blot Analysis—The MBP-M₂ mutant was solubilized with 1% Triton X100 at 4 mg/ml protein concentration. The solubilized fraction (30 μl) was subjected to SDS-PAGE on a 12% polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. After blocking with skim milk, the membrane was incubated with 0.05 μg/ml polyclonal antibodies against 6xHis, His-probe, followed by 0.4 μg/ml of goat anti rabbit IgG labeled with horseradish peroxidase. Bands were visualized by staining the membrane with dimethylaminobenzene.

Purification of the MBP-M₂ Mutant by Co²⁺-Imobilized Chelating Sepharose Gel and Cation Exchange Chromatography—Co²⁺-immobilized Chelating Sepharose gel was prepared by loading a 5× volume of 0.2 M CoCl₂ onto Chelating Sepharose gel. The solubilized MBP-M₂ mutant (100 μl), which was derived from 1 liter of culture and contained approximately 2 nmol of receptor, was loaded onto a 2 ml column volume of Co²⁺-immobilized Chelating Sepharose gel at 30 ml/h. The column was washed with 20 ml of a buffer containing 20 mM Tris-HCl, 500 mM NaCl, 0.1% digitonin, and 10 mM imidazole (pH 7.4) at the same flow rate. The receptor was eluted with 10 ml of a buffer containing 50 mM imidazole buffer (pH 7.0), 500 mM NaCl, and 0.1% digitonin. The eluted fraction was diluted 20-fold with a buffer containing 10 mM Mes-KOH (pH 6.0) and 0.1% digitonin and loaded onto a 0.5 ml column volume of SP-Sepharose at approximately 30 ml/h. The column was washed with 5 ml of a buffer containing 10 mM Mes-KOH (pH 6.0), 50 mM NaCl, and 0.1% digitonin, and eluted with 2.5 ml of a buffer containing 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 0.1% digitonin. The peak fraction was collected.

The M₂ mutant was purified by Co²⁺-immobilized Chelating Sepharose gel essentially by the same method as above. The expression of the M₂ mutant in Sf9/baculovirus and the isolation of the membrane fraction were performed by means of the methods previously described (5).

Reconstitution of the MBP-M₂ Mutant and G Protein G₁ or Gα—The reconstitution method was essentially the same as that previously reported (23, 35). G proteins were purified from porcine brain as described previously (36). Phosphatidylcholine (PC), phosphatidylinositol (PI), and cholesteryl hemisuccinate or PC and PI were dissolved in a chloroform and methanol solution [2:1 (v/v)] at 12:12:1 (w/w) or 1:1, respectively. The chloroform and methanol were evaporated off under constant N₂ aeration, and the resulting lipid film was suspended in a buffer containing 20 mM Hepes-KOH (pH 8.0), 1 mM EDTA, 160 mM NaCl (HEN), and 1% sodium cholate (Wako) at 4 mg/ml total lipid concentration. The purified MBP-M₂ mutant or the M₂ mutant (10 pmol) was mixed with 75 to 100 pmol of G, or Gα (10 μM), 0.7% CHAPS in HEN supplemented with 20 mM MgCl₂, 10 mM DTT, 2 μg/ml of the lipid mixture and 1 mM carbamylcholine. The mixture (200 μl total volume) was run through Sephadex G50 pre-equilibrated with HEN. The void volume fraction was collected, and used as the reconstituted vesicles.

[³H]QNB and [³²P]GTPyS Binding Assaying of the Reconstituted Receptor—The reconstituted vesicles were incubated with 1.5 nM [³H]QNB at 30°C in HEN containing 10 mM MgCl₂ and 5 mM DTT with or without 100 μM GTP in the presence of various concentrations of carbamylcholine or atropine in a 1 ml total reaction volume. For the [³²P]GTPyS binding assay, the vesicles were incubated in HEN containing 10 mM MgCl₂, 1 mM DTT, 50 nM [³²P]GTPyS, and 5 μM GDP in the presence of 1 mM carbamylcholine or 10 μM atropine at 30°C for 0, 2, 5, 10, or 20 min. The total reaction volume was 100 μl. The reaction was stopped by the addition of 500 μl of HEN containing 100 μM GTP. In both the [³H]QNB and [³²P]GTPyS binding assays, the vesicles after the reaction were trapped on GF/B glass fiber filters and then the radioactivity was measured with a liquid scintillation counter.

RESULTS

Expression of the MBP-M₂ Mutant—The expression levels of various fusion receptors are summarized in Table I. The M₂ mutant, rather than the wild type M₂ receptor, has been expressed because the wild type M₂ receptor tends to undergo degradation (most likely at the third intracellular loop region) during the purification process. Two strains of E. coli, BL21 and DH 5, were examined for expression of the MBP-M₂ mutant under the same culture conditions. The expression level of the MBP-M₂ mutant was 1.5-fold higher in BL21 than in DH 5, so we used BL21 throughout this work. The cells were grown at 37°C to a late growth

<table>
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<tr>
<th>Constructs</th>
<th>Promoters</th>
<th>Induction temperature (°C)</th>
<th>[³H]QNB binding pmol/100 μl culture</th>
<th>Western blotting</th>
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<tr>
<td>MBP-M₂ mutant</td>
<td>lac</td>
<td>20</td>
<td>220 ± 40 (3)</td>
<td>+</td>
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<tr>
<td></td>
<td></td>
<td>25</td>
<td>115 ± 14 (2)</td>
<td>N.D.</td>
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<td></td>
<td></td>
<td>37°</td>
<td>3 ± 0 (2)</td>
<td>N.D.</td>
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<tr>
<td>MBP-M₂ mutant—Trx (+His)</td>
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<td>188 ± 15 (2)</td>
<td>+</td>
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<td>MBP-M₂ mutant—Trx (−His)</td>
<td>lac</td>
<td>20</td>
<td>202 ± 32 (2)</td>
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<tr>
<td>MBP-M₂ mutant+Trx**</td>
<td>lac</td>
<td>20</td>
<td>200 ± 10 (2)</td>
<td>+</td>
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<tr>
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<td>20</td>
<td>92 ± 16 (2)</td>
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<tr>
<td>pelB-MBP-M₂ mutant</td>
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<td>20</td>
<td>85 ± 14 (2)</td>
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<td>20</td>
<td>0</td>
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<tr>
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<tr>
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<td>lac</td>
<td>20</td>
<td>0</td>
<td>+</td>
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phase (OD_{600} = 1.4), and incubated with 0.5 mM IPTG at 20°C for 24 h to induce the expression of the MBP-M\_ mutant. This culturing condition gave the best yield of the MBP-M\_ mutant among the conditions examined, such as those on induction of the expression at a lower cell density and for a longer time (Fig. 2). The membrane fraction derived from 100 ml of culture exhibited 0.2 nmol [\textsuperscript{3}H]QNB binding sites (6 pmol/mg of protein). The expression level of the MBP-M\_ mutant decreased to 50% or less when the cells were treated with 0.5 mM IPTG at 25°C instead of 20°C. Very little [\textsuperscript{3}H]QNB binding activity (less than 5 pmol in 100 ml of culture) was observed when the cells were treated with 0.5 mM IPTG at 37°C for 4 h. The co-expression of Trx or 6xHis and Trx to the C-terminus of the MBP-M\_ mutant was also not effective in increasing the expression level. This is in contrast with what is observed in the case of the neurotensin receptor, whose expression level was reported to increase on fusion of Trx to the C-terminus (17).

The expression level of the MBP-M\_ mutant under the T7 promoter in the BL 21(DE3) strain was 2-fold lower compared to that of the MBP-M\_ mutant under the lac promoter in the BL 21 strain. The expression of the MB\_ mutant without MBP fusion was not detected in the [\textsuperscript{3}H]QNB binding assay for the membrane preparation of E. coli. The expression of the MBP-M\_ mutant was further verified by Western blotting with polyclonal antibodies against 6xHis. A clear band appeared approximately at the position of the expected molecular weight (approximately 80 kDa) (Fig. 3).

Solubilization and Purification of the MBP-M\_ Mutant from E. coli and the M\_ Mutant from Sf9 Cells—The MBP-M\_ mutant was solubilized with a buffer containing 1% digitonin and 0.3% sodium cholate at the protein concentration of 2 mg/ml. Eighty to ninety percent of the MBP-M\_ mutant was extracted from the membrane fraction, as assessed as [\textsuperscript{3}H]QNB binding activity. The MBP-M\_ mutant was purified on Co\^2+-immobilized Chelating Sepharose gel and SP-Sepharose. Twenty-five to 40% of the [\textsuperscript{3}H]QNB binding activity in the solubilized fraction was recovered after the purification. The MBP-M\_ mutant was purified 100-fold and the specific activity increased from 5.0-6.5 to 420-650 pmol/mg protein. The M\_ mutant expressed in Sf9 cells was purified by the same method with a similar recovery (30-40% of the [\textsuperscript{3}H]QNB binding activity in the solubilized fraction), and the specific activity increased from 50-68 to 4,500-6,500 pmol/mg of protein.

Ligand Binding Properties of the MBP-M\_ Mutant Compared with the M\_ Mutant—Ligand binding properties were compared between solubilized preparations of the MBP-M\_ mutant and the M\_ mutant, which were derived from E. coli and Sf9 cells, respectively. Both the MBP-M\_ mutant and the M\_ mutant showed high affinity for [\textsuperscript{3}H]QNB and [\textsuperscript{3}H]INMS (Fig. 4). From Scatchard plots, the K\_d and B_{max} values for [\textsuperscript{3}H]QNB and [\textsuperscript{3}H]INMS were calculated for the MBP-M\_ mutant and the M\_ mutant (Table II). Figure 5 shows displacement curves of [\textsuperscript{3}H]QNB binding for carbachol (muscarinic agonist) and atropine (muscarinic antagonist). The K\_d values for carbachol and atropine were calculated from the displacement curve (Table II). Both the K\_d and K\_i values were very similar for the MBP-M\_ mutant and the M\_ mutant. These results indicate that the MBP-M\_ mutant expressed in E. coli has the same ligand binding activity as the M\_ mutant expressed in Sf9 cells, and that the addition of MBP at the N-terminus of the M\_ mutant does not alter the ligand binding properties.

Interaction of the MBP-M\_ Mutant with G Protein G\_2 and G\_1—We examined the ability of the MBP-M\_ mutant to interact with G protein G\_2 and G\_1. The MBP-M\_ mutant was reconstituted with purified G protein G\_2 and G\_1 in the dimer form.
lipid vesicles consisting of PC, PI, and cholesteryl hemisuccinate. Figure 6 shows displacement curves for carbamylcholine or atropine as to [^3H]QNB binding of the reconstituted vesicles in the presence or absence of 0.1 mM GTP. For both the MBP-M\(_4\) mutant and the M\(_4\) mutant, the displacement curves with carbamylcholine were biphasic in the absence of GTP and monophasic in the presence of GTP. The displacement curves with atropine for both the MBP-mutant and the M\(_4\) mutant were monophasic in either the presence or absence of GTP. The displacement curves with carbamylcholine in the absence of GTP well fitted the equation for a two-binding site model whereas the rest of the displacement curves well fitted the equation for a one-binding site model. The percentages and \(K_i\) values of the high and low affinity sites were calculated from the displacement curves (Table III). The results are consistent with the previous data obtained on reconstitution of G proteins with the M\(_4\) muscarinic receptor purified from porcine atria (37, 38) or the M\(_4\) mutant from SF9 cells (10), and can be explained with the same assumption; the M\(_4\) muscarinic receptor interacts with G\(_o\) in the absence of GTP and the M\(_4\)-G\(_o\) complex exhibits higher affinity for carbamylcholine than the free M\(_4\) receptor, whereas M\(_4\)-G\(_o\) and M\(_4\) exhibit the same affinity for atropine. The similarity in the proportions of high affinity sites in the absence of GTP indicate that the MBP-M\(_4\) mutant from E. coli has the same ability to couple with G protein G\(_o\) as the M\(_4\) mutant from SF9 (10). In addition, the similarity in \(K_i\) values of carbamylcholine and atropine indicates that the MBP-M\(_4\) mutant expressed in E. coli and the M\(_4\) mutant from SF9 cells have essentially the same characteristics as to interaction with both ligands and G proteins.

The MBP-M\(_4\) mutant was then examined as to its ability to activate G\(_o\) by measuring the agonist-dependent [^35S]GTP\(_\gamma\)S binding activity for the reconstituted vesicles (Fig. 7). The vesicles were incubated with 50 nM [^35S]GTP\(_\gamma\)S in

<table>
<thead>
<tr>
<th>TABLE II. Affinity of [^3H]QNB and [^3H]NMS for the MBP-M4 mutant and the M4 mutant. The (K_i) and (B_{max}) values for [^3H]NMS and [^3H]QNB were calculated from Scatchard plots for the MBP-M4 mutant and the M4 mutant in Fig. 4.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_i) (pM)</td>
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<tr>
<td>[^3H]QNB</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
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<tr>
<td>MBP-M(_4) mutant (E. coli)</td>
</tr>
<tr>
<td>M(_4) mutant (SF9)</td>
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Fig. 4. Binding of [^3H]QNB and [^3H]NMS with the MBP-M\(_4\) mutant from E. coli and the M\(_4\) mutant from SF9 cells. The E. coli (•, solid lines) and SF9 (O, dotted lines) membrane fractions were solubilized with 1% digitonin/0.3% sodium cholate at 2 mg/ml protein concentration. The solubilized fraction of the M\(_4\) mutant from SF9 was diluted 10-fold to adjust the receptor concentration to that of the MBP-M\(_4\) mutant. Ten microliters each of these fractions was incubated with various concentrations of [^3H]NMS (a-1) or [^3H]QNB (a-2) in 200 \(\mu\)l of a buffer containing 20 mM potassium phosphate buffer (pH 7.0), 0.1 M NaCl, and 0.1% digitonin at 30°C for 1 h. Each point represents the average of values determined in duplicate in two different experiments (b-1 and b-2). Scatchard transformation of data in (a-1) and (a-2).
the presence of 5 μM GDP and carbamylcholine or atropine. For both the MBP-M₄ mutant and the M₄ mutant, the extent of [⁴²S]GTP·S binding was greater in the presence of carbamylcholine than in the presence of atropine. These results are also consistent with the previous ones obtained for the M₄ receptor derived from mammalian tissues (37, 38) or Sf9 cells (10), and indicate that the agonist-bound MBP-M₄ mutant can facilitate the binding of [⁴²S]GTP·S to Gₒ in the presence of GDP in the same way as the M₄ mutant.

The MBP-M₄ mutant was also shown to interact with and activate G protein Gₒ. Figure 8a shows the [⁴²H]QNB binding activity of the reconstituted vesicles of the MBP-M₄ mutant and Gₒ in the presence of various concentrations of carbamylcholine and atropine, and in the presence or absence of 0.1 mM GTP. As with G protein Gₒ, the displacement curve with carbamylcholine in the absence of GTP well fitted the equation for a two-binding site model whereas the rest of the displacement curves well fitted the equation for a one-binding site model. The percentages and Kᵣ values of the high and low affinity sites were calculated from the displacement curves (Table III). The ability of the MBP-M₄ mutant to activate Gₒ was also demonstrated as stimulation by carbamylcholine of the [⁴²S]GTP·S binding activity of the reconstituted vesicles in the presence of GDP (Fig. 8b).

![Figure 9](http://example.com/figure9.png)

**Figure 9** shows the displacement curves for GDP of [⁴²S]GTP·S binding in the presence of carbamylcholine or

![Graph](http://example.com/graph.png)

**Fig. 6. Interaction of the MBP-M₄ mutant and the M₄ mutant with G protein Gₒ.** The MBP-M₄ mutant (a) and the M₄ mutant (b) were solubilized from E. coli and Sf9 membranes, respectively, purified, and then reconstituted into lipids with Gₒ purified from porcine brain. The reconstituted vesicles containing 100 fmol of the MBP-M₄ mutant or the M₄ mutant in a 50 μl volume were incubated with 950 μl of buffer containing 1.5 nM [⁴²H]QNB in the presence of various concentrations of GTP and agonists. The graphs shown are each representative of four experiments.

**TABLE III. Affinity of carbamylcholine and atropine for the MBP-M₄ mutant and the M₄ mutant reconstituted with G proteins.** The Kᵣ values for carbamylcholine and atropine were calculated from the data in Figs. 6, 7, and 9. Abbreviations: (H), high-affinity binding site; (L), low-affinity binding site.

<table>
<thead>
<tr>
<th>Protein</th>
<th>GTP(–)</th>
<th>GTP(+)</th>
<th>Kᵣ (carbamylcholine) GTP(–)</th>
<th>GTP(+)</th>
<th>Proportion of high-affinity sites (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP-M₄ mutant-Gₒ</td>
<td>0.69</td>
<td>0.85</td>
<td>15.3</td>
<td>7.99</td>
<td>4.34</td>
</tr>
<tr>
<td>M₄ mutant-Gₒ</td>
<td>0.84</td>
<td>0.59</td>
<td>18.9</td>
<td>10.0</td>
<td>6.60</td>
</tr>
<tr>
<td>MBP-M₄ mutant-Gₒ</td>
<td>—</td>
<td>—</td>
<td>21.5</td>
<td>6.76</td>
<td>14.1</td>
</tr>
<tr>
<td>MBP-M₄ mutant-Gₒ</td>
<td>0.77</td>
<td>0.65</td>
<td>134.0</td>
<td>7.43</td>
<td>7.2</td>
</tr>
</tbody>
</table>

![Graph](http://example.com/graph2.png)

**Fig. 5. Displacement of [⁴²H]QNB by carbamylcholine and atropine.** The experimental conditions were the same as those given in the legend to Fig. 3, except that a single concentration of [⁴²H]QNB (1.5 nM), and different concentrations of GTP and agonists were incubated with the solubilized MBP-M₄ mutant (●, ○, solid lines) and M₄ mutant (△, □, dotted lines). The graphs shown are each representative of four experiments. The actual value for 100% is 5,000 to 6,000 dpm.
Fig. 7. Stimulation of [35S]GTPyS binding to G protein Go reconstituted with the MBP-M2 mutant or the M1 mutant. Gm and the MBP-M2 mutant from E. coli (a) or the M1 mutant from SF9 (b) were reconstituted into lipids as described in the legend to Fig. 5. The reconstituted vesicles containing 100 fmol of the MBP-M2 mutant or the M1 mutant were incubated with 50 nM [35S]GTPyS and 5 μM GDP in the presence of 1 mM carbamylcholine (○) or 10 μM atropine (●). The graphs shown are each representative of four experiments.

Fig. 8. Interaction of the MBP-M2 mutant with and stimulation of [35S]GTPyS binding to G protein Ga. The MBP-M2 mutant was reconstituted into lipids with Ga purified from porcine brain. The reconstituted vesicles containing 100 fmol of the MBP-M2 mutant or the M1 mutant in a 50 μl volume were incubated with 950 μl of buffer containing 1.5 nM [3H]QNB in the presence of carbamylcholine (○, ●) or atropine (▲, ▲), and in the presence (▲, ●) or absence (○, ▲) of 0.1 mM GTP at 30°C for 1 h. Curve fitting was performed as described in the legend to Fig. 5 (a). The reconstituted vesicles containing 100 fmol of the MBP-M2 mutant were incubated with 50 nM [35S]GTPyS and 5 μM GDP in the presence of 1 mM carbamylcholine (○) or 10 μM of atropine (●) (b). The graphs shown are each representative of three experiments.

Effect of Cholesterol on the Interaction of the MBP-M2 Mutant with G Protein Ga—The MBP-M2 mutant and G protein Ga were reconstituted into lipid vesicles without cholesteryl hemisuccinate. The displacement of [3H]QNB binding by carbamylcholine was assessed in the same way as described previously. The displacement curve in the presence of GTP well fitted the equation for a one-binding site model, whereas that in the absence of GTP well fitted the equation for a two-binding site model (Fig. 10a). The percentages and Kᵢ values of the high and low affinity sites were calculated from the displacement curves (Table III). The similarity in the percentages and Kᵢ values showed that the MBP-M2 mutant reconstituted into cholesterol-containing vesicles and cholesterol-free vesicles had the same ability to associate with G protein Ga.

The reconstituted vesicles were also examined as to their ability to bind [35S]GTPyS in the presence of carbamylcholine or atropine as described previously. In both cholesterol-containing (Fig. 7a) and cholesterol-free vesicles (Fig. 10b), the extent of [35S]GTPyS binding was greater in the presence of carbamylcholine (○) or 10 μM atropine (●) in the presence of atropine. These results indicate that cholesterol is not required for the MBP-M2 mutant to interact with and activate G protein Ga.

DISCUSSION
In this study, we have shown that the MBP-M2 mutant expressed in E. coli has the ability to interact with both muscarinic ligands and G proteins Ga and Gm.

Fusion of MBP was necessary for functional expression. The MBP could not be replaced by GST or a leader sequence, pelB. Expression of the pel B-M2 mutant was not detected on Western blotting while expression of the GST-
M2 mutant was detected on Western blotting, but no [3H]QNB binding activity was detected. These results suggest that fusion of a periplasmic protein, MBP, but not a cytosolic protein, GST, is required for the membrane translocation and proper folding of the M2 receptor in membranes. MBP is secreted in a SecB-dependent manner and the MBP fusion receptor is thought to manipulate this secretional machinery to translocate itself to the E. coli inner membrane (30). The expression level was sensitive to the induction temperature; a low induction temperature is critical for expression of a great amount of functional receptors. This is consistent with the suggestion of Grishammer and Tate that slow transcription is essential for proper folding of the M2 receptor in membranes. MBP is secreted in a SecB-dependent manner and the inducible expression of the MBP-M2 mutant under the T7 promoter (PET25b) was half that of the MBP-M2 mutant under the lac promoter when cells were cultured under the same conditions. The lac promoter is a much weaker promoter than the T7 promoter. The expression level was sensitive to the lac induction temperature; a low induction temperature is critical for expression of a great amount of functional receptors. This is consistent with the suggestion of Grishammer and Tate that slow transcription is essential for proper folding of the MBP-neurotensin fusion receptor into membranes (19).

Other GPCRs such as the substance K receptor (40) and the M1 muscarinic receptor (18) have been reported to be expressed functionally in terms of ligand binding activity on fusion of MBP to their N-termini. To our knowledge, however, the MBP fusion receptors including the neurotensin receptor have not been examined as to their ability to interact with G proteins. In the present study, we solubilized and partially purified the MBP-M2 mutant, and reconstituted it with purified G protein Gs or Gi, as described in the legend to Fig. 5. The reconstituted vesicles containing 100 fmol of the MBP-M2 mutant were incubated with 50 nM [35S]GTPyS (15,000 cpm/50 µl reaction buffer) and various concentrations of GDP in the presence of 1 mM carbamylcholine (O, △) or 10 µM atropine (○, ▲) at 30°C for 30 min. The graphs shown are each representative of three experiments. (O, △) represent the data points for Gs, and (○, ▲) these for Gi.

M2 mutant expressed in S99 baculovirus system. We have already shown that the M2 mutant expressed in S99 cells has the same ability to interact with muscarinic ligands and G proteins as the wild type M2 muscarinic receptor purified from porcine atria (10). Thus, we conclude that the MBP-M2 mutant has the same ability as the mammalian M2 receptor. E. coli will be the simplest and the least expensive means of producing functionally active GPCRs.

Fig. 9. Displacement of [35S]GTPyS by GDP in the presence of carbamylcholine or atropine. The MBP-M2 mutant was reconstituted into lipids with G protein Gs or Gi, as described in the legend to Fig. 5. The reconstituted vesicles containing 100 fmol of the MBP-M2 mutant were incubated with 50 nM [35S]GTPyS (15,000 cpm/50 µl reaction buffer) and various concentrations of GDP in the presence of 1 mM carbamylcholine (O, △) or 10 µM atropine (○, ▲) at 30°C for 30 min. The graphs shown are each representative of three experiments. (O, △) represent the data points for Gs, and (○, ▲) these for Gi.

Fig. 10. Interaction of the MBP-M2 mutant with and stimulation of [35S]GTPyS binding to G protein Gs in the absence of cholesterol. The MBP-M2 mutant and Gs were reconstituted into lipids consisting of phosphatidylcholine and phosphatidylinositol (50:50 w/w) only. The experimental conditions were the same as those given in the legend to Fig. 5, except that cholesteryl hemisuccinate was omitted. Incubation was carried out in the presence (○) or absence (▲) of 0.1 mM GTP (a). The reconstituted vesicles containing 100 fmol of the MBP-M2 mutant were incubated with 50 nM [35S]GTPyS and 5 µM GDP in the presence of 1 mM carbamylcholine (O) or 10 µM atropine (▲) (b). The graphs shown are each representative of four experiments.

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by the lack of palmitoylation. Another possible explanation is that the unpalmitoylated receptor purified from Sf9 cells is less stable and is recovered in a lower yield on reconstitution compared to the palmitoylated receptor, whereas the unpalmitoylated receptor purified from E. coli is stable and recovered in the same yield as the palmitoylated receptor. In this respect, it should be noted that the degree of purification was ten times higher for the M2 mutant from Sf9 cells than the MBP-M2 mutant from E. coli, and that the highly purified receptor becomes more unstable during the reconstitution procedure than the less purified receptor. The present finding is compatible with the report that the function of the M2 mutant in Chinese hamster ovary (CHO) cells is not affected by the presence or absence of palmitoylation (42).

Lipids of E. coli membranes comprise 70% phosphatidylethanolamine, 15% phosphatidylglycerol, and 15% cardiolipin (w/w), and are different from those of mammalian or Sf9 cell membranes, particularly in that they do not contain cholesterol (29). The present results suggest that the function of the M2 receptor is not affected by lipid environment of membranes. Our results provide direct evidence that cholesterol is not required for the interaction of the M2 muscarinic receptor with muscarinic ligands and G proteins. Strictly speaking, we cannot exclude the possibility that cholesterol was copurified with G proteins which were purified from porcine brain. This, however, is not likely because G proteins are not integral membrane proteins with transmembrane segments.

In summary, we have shown that the functional M2 muscarinic receptor can be expressed in E. coli when MBP is fused to its N-terminus. The MBP-fused receptor possesses the same ligand binding activity and ability to interact with G proteins as the M2 muscarinic receptor expressed in Sf9 cells. We have also shown that cholesterol and palmitoylation are not essential for the function of the muscarinic receptor.

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Expression of Muscarinic Receptor in E. coli


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