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Engineering of a proteolytically stable human β_2-adrenergic receptor/maltose-binding protein fusion and production of the chimeric protein in *Escherichia coli* and baculovirus-infected insect cells

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Abstract

The hydrophobic human β_2 adrenergic receptor was produced in fusion to the hydrophilic maltose-binding protein (*Mal*E) in *Escherichia coli*. Photoaffinity labeling with the adrenergic ligand [^{125}I]cyanopindolole-diazirine indicated that the majority of the protein was proteolyzed in the intergenic region between the fusion partners after production in *E. coli*. The simple and fast genetics of the bacterium enabled us to engineer a linker with an increased proteolytic stability. The fusion protein produced in *E. coli* was fully functional with respect to binding of adrenergic ligands and coupling to stimulatory GTP-binding protein. The production level with 3 pmol receptor fusion protein per mg membrane protein in a crude membrane preparation was significantly higher than those reported for other β_2 adrenergic receptor constructs in *E. coli*. After solubilization with dodecanoyl sucrose, the fusion protein was purified to near homogeneity by affinity chromatography on immobilized Ni^{2+} ions (binding to a C-terminal His_6-tag) and on crosslinked amylose (binding to the MalE). In order to achieve higher production levels, the fusion protein preceded by an insect signal peptide was produced in baculovirus-infected insect cells. As expected, the production

Abbreviations: β_2AR, human β_2 adrenergic receptor; G protein, heterotrimeric GTP-binding protein; GPCR, G proteins-coupled receptor; MalE, maltose-binding protein.

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level with about 17 pmol receptor per mg membrane protein was higher in the insect cells than in *E. coli*. The receptor fusion protein produced in the insect cells bound adrenergic ligands and activated heterotrimeric GTP-binding proteins with biochemical properties comparable to that of the unfused receptor.

Keywords: Human β_2-adrenergic receptor; Maltose-binding protein; Fusion protein; Proteolytic stability; Membrane protein purification

1. Introduction

The β_2-adrenergic receptor (β_2AR) is probably the best characterized member of a large family of hormone and neurotransmitter receptors that mediate their function through interaction with heterotrimeric GTP-binding proteins (G proteins) (Strader et al., 1989; Collins et al., 1991; Dohlmann et al., 1991; Bourne, 1997). As for all G protein-coupled receptors (GPCRs), hydrophobicity analysis of the β_2AR predicts the presence of seven transmembrane helices connected by short hydrophilic loops with the N-terminus outside the cell. However, at present a high-resolution three-dimensional structure has not been reported for any of the G protein-coupled receptors. A prerequisite for structural determination by X-ray diffraction is crystallization which, particularly for membrane proteins, is difficult. Hydrophilic patches of the protein surface are important for the formation of stabilizing intermolecular contacts in the crystal since hydrophobic transmembrane regions are covered by detergent micelles (Ostermeier and Michel, 1997). Therefore, the crystallization probability of mainly hydrophobic proteins might be enhanced by enlarging the hydrophilic surface. One way to overcome the problem of hydrophilicity is the co-crystallization with hydrophilic antibody fragments (Ostermeier et al., 1995). Alternatively, one can produce the hydrophobic protein of interest as a fusion protein with a hydrophilic partner. Nevertheless, fusion of a membrane protein to another protein might result in proteolytic digestion of the fusion protein. In this study, we chose the hydrophilic maltose-binding protein (Duplay et al., 1984) from *Escherichia coli* (MalE) as a fusion partner for the human β_2AR. For the purpose of crystallizing the hydrophobic transmembrane receptor, a MalE fusion has several advantages: (1) As a water-soluble globular protein of about the same molecular mass MalE greatly enlarges the hydrophilic surface of the β_2AR. (2) The periplasmic protein MalE is secreted through the *E. coli* inner membrane and, as the N-terminal fusion partner, should enforce the correct topology of the β_2AR. (3) A high resolution X-ray structure of MalE is available (Spurlino et al., 1991) and should simplify structural analysis of the fusion protein. Additionally, high-affinity antibodies directed against MalE are available and MalE can be purified using amylose affinity chromatography (Ferenci and Klotz, 1978).

The human β_2AR has already been functionally expressed in a variety of different organisms including *E. coli* (Marullo et al., 1988, 1989; Breyer et al., 1990; Chapot et al., 1990) and baculovirus-infected insect cells (George et al., 1989; Reiländer et al., 1991). Although production levels reported in *E. coli* are low, it proves to be the organism in which genetic manipulations of recombinant proteins can be performed most quickly. In both expression systems addition of N- or C-terminal fusion partners did not alter the ligand- or G protein-binding properties of the receptor (Chapot et al., 1990; Kobilka, 1995; Kwatra et al., 1995). The G protein-linked receptor for neuropeptide Y has already been functionally expressed as a fusion with MalE in *E. coli* and was adequate for functional studies (Münch et al., 1995).

In this study, we report the engineering of a protease-stabilized MalEβ_2AR fusion protein and describe its pharmacological and biochemical properties after production in *E. coli* as well as in baculovirus-infected Sf9 insect cells.

2. Material and methods

2.1. Molecular cloning techniques and protein production

DNA isolation, restriction enzyme analysis, agarose gel electrophoresis, *Bal*31-digestion, DNA sequencing and cloning procedures were performed using established techniques (Sambrook et al., 1989). Expression studies were performed using *E. coli* XL1-Blue (Stratagene), JM83 (Yanish-Perron et al., 1985), TG1 (Sambrook et al., 1989), CAG627 (Spiro and Guest, 1987), and KS474 (Strauch et al., 1989). Plasmid pMal-p used for construction of the *E. coli* expression plasmids was purchased from New England Biolabs. The recombinant baculovirus vector was obtained by inserting the coding sequence for the MalEβARΔ132His fusion protein without its signal peptide from the *E. coli* vector into the *Xba*I/*Kpn*I restricted vector pVL92Mel (Lenhard et al., 1996) which encodes the prepromelittin signal peptide. The resulting plasmid codes for the insect signal peptide, a 12-amino acid spacer, and the MalEβAR fusion leaving the internal deletion of the original construct unchanged. After cotransfection of the recombinant plasmid with BaculoGold baculovirus DNA (Pharmingen), recombinant baculovirus was selected and the correctness of the virus was confirmed by Southern analysis. Plasmid pTF3 (ATCC accession number 57 537) was the original source of the human β_2AR cDNA (Kobilka et al., 1987). All constructs were ensured by DNA sequencing.

Bacteria were grown at 37°C in Luria Broth medium containing 150 μg ml^{-1} of ampicillin to an A_{600}-value of 0.4–0.8. Production of the fusion protein was induced by the addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) overnight at 22°C. The insect cell line Sf9 (*Spodoptera frugiperda*) was cultured in Sf1 medium (BioConcept, Umkirch, Germany) supplemented with 50 mg l^{-1} gentamycin. Cells were infected with the recombinant baculovirus as described (Summers and Smith, 1987).

2.2. Membrane preparation, fractionation and solubilization

E. coli cells were harvested by centrifugation, resuspended in 90 mM NaCl, 10 mM Tris pH 7.8, 0.5 mM PefablocSC (Serva) and passed twice through a French-pressure cell at 90 MPa (13 000 psi). Membranes were collected by centrifugation at 150 000 × *g* for 30 min, resuspended in the same buffer and kept at −20°C until use. Membrane fractionation was performed by sucrose-density centrifugation according to a recent report (Chapot et al., 1990). The protein concentrations were determined using the bicinchinonic protein assay reagent (Pierce, Rockford, IL). Routinely, solubilization of *E. coli* membranes was performed at a protein concentration of 1 mg ml^{-1} in borate buffer (0.5 M H_3BO_3 pH 9.0, 0.5 M NaCl, 0.5 mM Pefabloc SC) with 0.25–0.5% dodecanoyl sucrose (Calbiochem or Mitsubishi-Kasei Food Corporation, Tokyo, Japan) gently agitated for 30 min at 4°C. Non-solubilized protein was removed by centrifugation at 150 000 × *g* for 30 min. Membrane preparation of baculovirus-infected Sf9 cells was performed by nitrogen cavitation as described previously (Boege et al., 1988; Reiländer et al., 1991). Insect cell membranes were solubilized at a protein concentration of 5.5–8 mg ml^{-1} using 1% dodecanoyl sucrose in a buffer containing 20 mM imidazole, 150 mM NaCl, 100 mM Na-borate pH 9.0 supplemented with protease inhibitors (1 μM pepstatin, 10 μM leupeptin, 10 μM chymostatin, 1 μM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg ml^{-1} aprotinin).

2.3. Photoaffinity labeling and radioligand binding assays

E. coli membranes (200 fmol ligand-binding sites) were incubated with an equimolar amount of [^{125}I]cyanopindolole-diazirine (NEN) (Burgermeister et al., 1983) in 500 μl of 10 mM Tris pH 7.4, 5 mM $MgCl_2$, 2 mM $CaCl_2$ for 30 min at 30°C in the dark in the absence or presence of 10 μM (−)alprenolol. After addition of 900 μl buffer to the probe, the radioligand was photolyzed for 10 min using a 254 nm UV hand lamp.

Membranes were collected by centrifugation at 135 000 × *g* for 15 min. Proteins were separated on reducing 10% SDS-polyacrylamide gels and labeled receptor was visualized by autoradiography.

Cells, membranes, or solubilized receptor were incubated in 20 mM Hepes pH 7.4, 12 mM $MgCl_2$, 100 mM NaCl, 0.01% NaN_3 with 2 nM (−)-[^{3}H]CGP-12177 (NEN) in an assay volume of 200 μl for 30 min at 30°C in the absence or presence of 10 μM (−)alprenolol. Bound ligand was separated from free ligand by rapid vacuum filtration over Whatman GF/F filters presoaked in 0.3% polyethyleneimine and quantified by liquid scintillation counting. For saturation binding (−)-[^{3}H]CGP-12177 was used at various concentrations (20 pM to 5 nM). Competition binding experiments were carried out using 200 pM (−)[^{3}H]CGP-12177 and various concentrations of the indicated competitors (Sigma). K_D-values were calculated by non-linear regression or, for the competition experiments, from the IC_{50}-values. Dissociation kinetics were characterized after rapid 50-fold dilution of a pre-equilibrated [^{3}H]CGP-12177-receptor complex and dissociation and association constants were determined.

2.4. Gel electrophoresis, immunoblot analysis, carbohydrate analysis and electron microscopy

Proteins were electrophoresed on 10% SDS-polyacrylamide gels and directly stained with silver or blotted onto an Immobilon-P membrane (Millipore) and probed with a MalE-specific polyclonal (1:10 000) antibody (New England Biolabs) or a monoclonal antibody directed against a hexahistidine tail (1:1000; Dianova). Visualization was performed with an appropriate alkaline phosphatase-coupled secondary antibody and the respective chromogenic substrates.

For protein deglycosylation, 2×10^5 infected Sf9 cells were lyzed in 1% SDS and DNA was degraded by incubation with Benzonase (Merck) on ice for 1 h. Deglycosylation was performed using peptide *N*-glycosidase F (1250 U, New England Biolabs) at 37°C in the presence of 2% Nonidet P-40. Tunicamycin was supplemented at a concentration of 20 μg ml^{-1} to the culture medium of the Sf9 cells 1 day after infection.

For electron microscopy, infected Sf9 cells were fixed in situ with 4% paraformaldehyde/0.1% glutardialdehyde in 0.1 M PBS, pH 7, for 2 h at room temperature. After washing in PBS, the cells were treated with 2% glycine in PBS (15 min). For pre-embedding immunostaining the cells were incubated with 1% bovine serum albumine in PBS, with 0.1% bovine serum albumine in PBS and then with the polyclonal anti-MalE antiserum (1:500) at 4°C overnight. Subsequently, after washing with 0.1% bovine serum albumine in PBS, the cells were incubated with the secondary goat anti-rabbit antibody (diluted 1:20 in PBS) coupled to 10 nm gold particles (Amersham-Buchler) for 90 min. After immunogold labeling the cells were refixed in 1% glutardialdehyde in PBS for 30 min, washed with PBS, enclosed in 4% agar–agar, postfixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer and stained en bloc with 1% uranyl acetate. The samples were dehydrated with ethanol, embedded in Spurr's resin (Spurr, 1969) and polymerized at 70°C. Afterwards, the sections were double stained with 2% uranyl acetate and lead citrate and viewed in an electron microscope (Philips EM CM12).

2.5. G-Protein coupling and adenylate cyclase assay

Functional reconstitution of solubilized *E. coli*-derived MalEβAR fusion protein with pure G_S-protein isolated from turkey erythrocytes in lipid vesicles and analysis of G_S-dependent stimulation of adenylate cyclase prepared from rabbit myocardium was performed as described earlier (Pfeuffer and Metzger, 1982; Hekman et al., 1984; Feder et al., 1986). The concentration of cAMP was measured according to the method of Salomon et al. (1974). For analysis of the fusion protein from insect cells, Sf9 cells were coinfected with baculoviruses encoding the MalEβAR fusion protein and the $G_{\alpha s}$, $G_{\beta 1}$, and $G_{\gamma 2}$ subunits of the human G protein (Iniguez-Lluhi et al., 1992; Linder et al., 1993; Grünewald et al., 1996). Three days post infection, the Sf9 membranes were solubilized and reconstituted into phosphatidylcholine vesicles by detergent removal with biobeads SM-2 (Bio-Rad) which were subsequently fused to larger

vesicles with 50% polyethyleneglycol. [^{35}S]GTPγS binding to the vesicles in the presence or absence of 100 μM (−)isoproterenol or 1 mM (±)-alprenolol was determined.

2.6. Protein purification

Solubilized *E. coli* membranes (17 mg of protein) were applied to 3 ml Ni^{2+} charged Sepharose Fast Flow (Pharmacia) equilibrated in purification buffer (50 mM boric acid, pH 9.0, 150 mM NaCl, 5% (w/v) glycerol, 0.05% dodecanoyl sucrose and 2 mM phenylmethylsulfonyl fluoride at a flow rate of 0.33 ml min^{-1}. Unspecific binding was prevented by the addition of 20 mM imidazole. After washing (20 mM imidazole), specific elution was achieved with 100 mM imidazole. Fractions containing receptor were pooled and applied to 2 ml of amylose resin (New England BioLabs) at a flow rate of 0.25 ml min^{-1}. The resin was washed with purification buffer and the fusion protein was eluted with 20 mM maltose. Subsequently, the receptor was concentrated in a centricon by a factor of ≈ 50 without substantial loss of binding activity.

Solubilized insect cell membranes (100 mg of protein at a concentration of 10 mg ml^{-1}) were applied to the Ni^{2+} charged Sepharose Fast Flow and, after washing (20 mM imidazole), elution was performed with 250 mM imidazole. Amylose affinity chromatography was performed as described above but with the addition of 1 mM EDTA to all buffers. Alternatively, receptor-containing fractions from the immobilized metal ion chromatography (IMAC) were applied to alprenolol sepharose (Caron et al., 1979). Elution was performed in 20 mM Tris pH 8.0, 150 mM NaCl, 5% glycerol, 2 mM EDTA, 1 mM alprenolol, 0.05% dodecanoyl sucrose at 37°C. The eluate was analyzed by ligand-binding analysis after removal of alprenolol on HiTrap gel filtration columns (Amersham Pharmacia Biotech, Freiburg, Germany).

3. Results

3.1. Construction of a MalEβAR fusion protein and its production in E. coli

Plasmid pMalEXaβAR was constructed by inserting a 1.8-kb *Xba*I/*Hin*dIII DNA fragment encoding the human β_2AR into the vector pMal-p (Fig. 1a,b, top bar). The resulting plasmid should induce the production of a MalEβAR fusion protein with a molecular mass of approximately 89 kDa (Fig. 1a). Most of the intergenic multiple cloning site of the vector was still present between the coding regions of the two fusion partners. After transformation into the *E. coli* strain XL1-Blue and induction with IPTG, recombinant clones showed a time-dependent increase of binding sites as measured with the hydrophilic adrenergic ligand [^{3}H]CGP-12177, thus, indicating production of intact fusion protein on the cell surface. As expected, *E. coli* XL1-Blue transformed with vector pMal-p did not reveal any significant binding. Maximal production level of 2 pmol receptor per mg of protein was obtained at 22°C whereas at higher temperatures a drastic decrease in binding sites was observed. Furthermore, the receptor yield was not increased by the use of other *E. coli* strains like JM83 or TG1. Photoaffinity labeling with [^{125}I]cyanopindolole-diazirine resulted in labeling of a 89- and a 45-kDa protein (Fig. 2a, lane 8). The incorporation of label was inhibited by addition of 10 μM (−)alprenolol. Since the apparent molecular masses of the photolabeled proteins were in the range of the calculated molecular masses of the MalEβAR fusion protein (≈ 89 kDa) and the receptor alone (≈ 45 kDa), we concluded that the fusion protein had been cleaved in the flexible and, therefore, accessible linker region by endogenous proteases. This conclusion was further confirmed by a production study using plasmid pMalEXaβ_2AR + 39 (Fig. 1b, bar 2). Due to a cloning artefact this construct contained an extended linker region between the two fusion partners. Here, after production of the respective fusion protein, only the proteolyzed protein of ≈ 45 kDa was labeled by the photoaffinity ligand (data not shown).

3.2. Engineering of a protease-stabilised MalEβAR fusion protein

In order to prevent proteolytic attack in the linker region of the fusion protein, the protease-deficient *E. coli* strains KS747 and CAG627 were

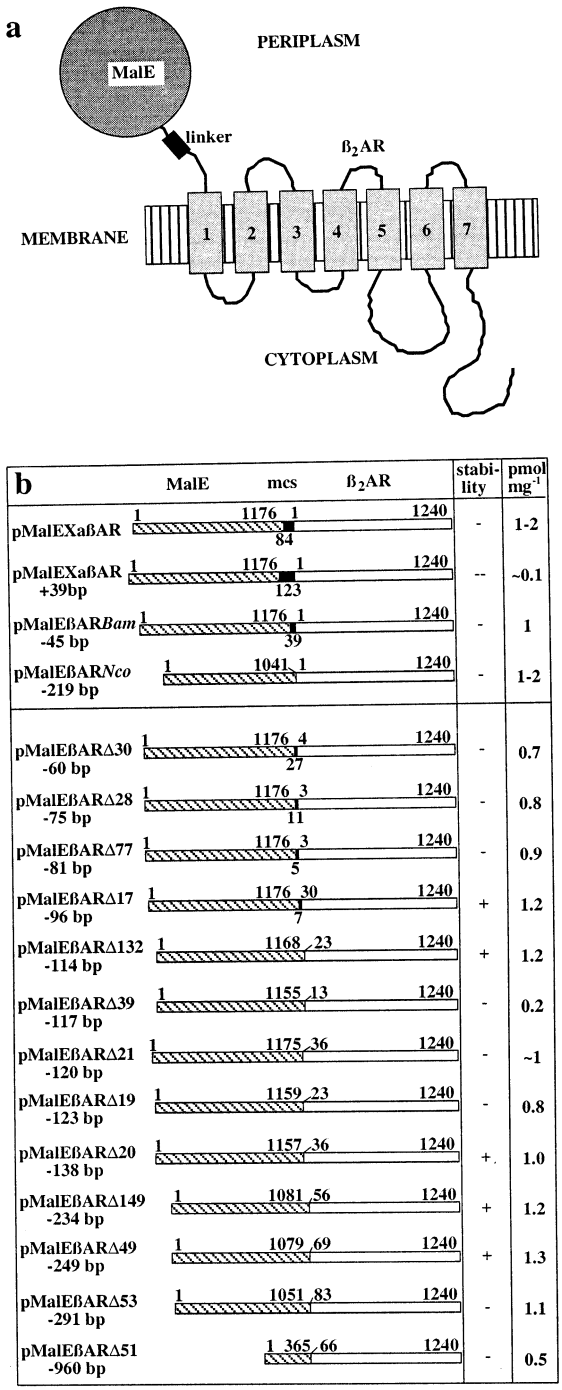

Fig. 1.

used for production. However, subsequent photoaffinity labeling revealed that the MalEβAR fusion protein was still proteolyzed (data not shown). In another approach, the linker region encoded in plasmid pMalEXaβAR was partially deleted by restriction with either *Bam*HI or *Nco*I and subsequent religation (pMalEβAR*Bam*, pMalEβAR*Nco*; Fig. 1b, bars 3 and 4). Production from the resulting plasmids showed about the same number of ligand-binding sites as the original one but the stability of the resulting fusion proteins was, if at all, only slightly improved (Fig. 2a, lanes 6 and 7). Subsequently, plasmid derivatives of pMalEβAR*Bam* with a shortened intergenic region were generated by a random approach using exonuclease *Bal*31 (Fig. 1b, lower part). From 200 clones analysed, 30 clones showed drastically reduced growth rates after induction and also exhibited specific and time-dependent binding of [^{3}H]CGP-12177. As expected, DNA sequencing revealed that the plasmids isolated from these clones had 'in frame' deletions not affecting the first putative transmembrane region of the receptor. As was detected by photoaffinity labeling, five out of the 30 clones showed an increased proteolytic stability of the fusion proteins. As can be seen in Fig. 2 (lanes 1–5), here only the 89-kDa protein was labeled by

Fig. 1. Schematic representation of various MalEβAR fusion protein constructs for heterologous production in *E. coli*. The proposed topology of the fusion protein encoded by the original construction is given in (a). The schematic representations of the coding regions are presented in (b). The first four bars represent the initial plasmid pMalEXaβAR, plasmid pMalEXaβAR + 39 and the plasmids pMalEβAR*Bam* and pMalEβAR*Nco* which were derived from plasmid pMalEXaβAR after cleavage with *Bam*HI and *Nco*I, respectively, and religation. In the lower part, 13 constructs which resulted from the random approach using *Bal*31 are drawn. Here, plasmid pMalEβAR*Bam* after restriction with *Bam*HI was treated with *Bal*31 for different times and subsequently religated. Under the names of the plasmids, the number of deleted basepairs is given. In the two columns at the right side, the proteolytic stability of the respective fusion proteins as well as the production level as determined by [^{3}H]CGP-12177 binding in pmol mg^{-1} of total *E. coli* protein is indicated. Basepair-numbering of the *Mal*E- (diagonal lines) and the β_2AR-gene (open bar) begins at the start codons. The number of nucleotides in the linker region is given below the black part of the bars.

[^{125}I]cyanopindolole-diazirine. Nevertheless, immunoblot analysis performed with polyclonal antibodies directed against MalE revealed the presence of truncated fusion protein (Fig. 2b, lanes 1–5). From the fact that these proteolytic fragments were not labeled by [^{125}I]cyanopindolole-diazirine, one can conclude that here, the β_2AR part of the fusion proteins was the target of proteolytic action. From the five clones, the clone producing the MalEβARΔ132 fusion protein was chosen for further examination (Fig. 1b, lower part).

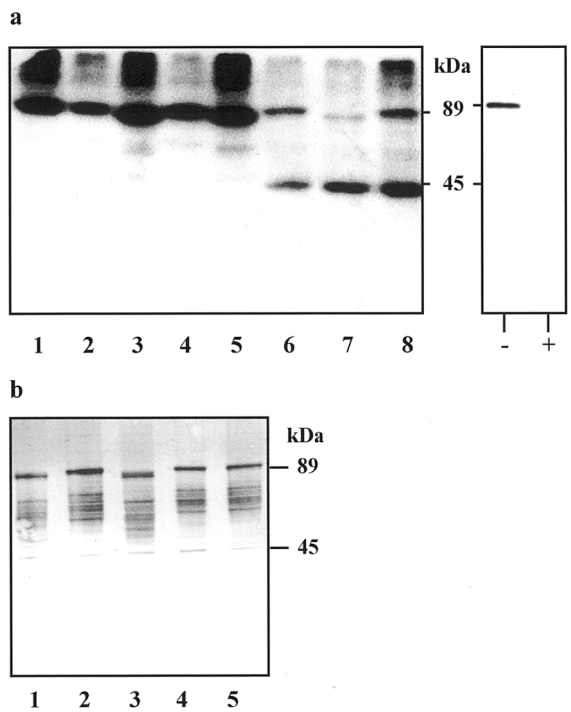

Fig. 2. Biochemical analysis of different MalEβAR fusion proteins after production in *E. coli*. (a) Photoaffinity labeling with [^{125}I]cyanopindolole-diazirine of membranes prepared from *E. coli* XL1-Blue transformed with (1) pMalβARΔ17, (2) pMalβARΔ20, (3) pMalβARΔ49, (4) pMalβARΔ132, (5) pMalβARΔ149, (6) pMalβAR*Bam*, (7) pMalβAR*Nco*, and (8) pMalEXaβAR. The 89-kDa band represents the fusion protein; the apparent molecular mass of the unfused β_2-adrenergic receptor is about 45 kDa. Labeling was inhibited in the presence of alprenolol in all cases, here only shown for the fusion protein produced from plasmid pMalβARΔ132 ($-$ in the absence, $+$ in the presence of 10 μM ($-$)alprenolol). (b) Immunoblot analysis of the stabilized fusion proteins using anti-MalE antibodies. The clone numbers are identical to that in (a). For better visualization of degradation products the immunoblot was over-developed.

3.3. Characterization of the MalEβAR fusion protein produced in E. coli

For membrane preparation, cells were disrupted in a French pressure cell at 90 MPa. In contrast to Hill and Sillence (1997) who reported a recovery of about 13% receptor at a working pressure of 6.9 MPa, the yield in our hands reached up to 95%. Further increase in working pressure resulted in a more complete cell disruption but on the other hand led to a dramatic loss of functional receptor. Therefore, the lower working pressure was used routinely. The low spin centrifugation step was omitted, resulting in accumulation of 95% of the binding sites and 40% of the total protein in the high spin 'membrane' pellet. Here, the specific activity was about 3 pmol mg^{-1} protein. After sucrose density centrifugation, 96% of the binding sites were detected in the bacterial inner-membrane fraction where the receptor fusion accumulated to about 6 pmol mg^{-1} membrane protein (data not shown). Specific binding of [^{3}H]CGP-12177 to membranes prepared from *E. coli* bearing plasmid pMalEβARΔ132 was saturable and of high affinity ($K_D = 96 \pm 9$ pM) with Scatchard analysis indicating the presence of a single, homogeneous binding site (Fig. 3a). In competition binding experiments, the K_i values for alprenolol, ($-$)-isoproterenol and ($+$)isoproterenol were determined to be 0.49 ± 0.05 nM, 58.5 ± 7.3 nM and 4.7 ± 1.8 μM, respectively (data not shown). After solubilization of the membranes with dodecanoyl sucrose the fusion protein was reconstituted into vesicles supplemented with purified G_S protein from turkey erythrocytes. In the presence of GTPγS, addition of the β_2AR agonist ($-$)-isoproterenol to the vesicles led to irreversible activation of G_S protein as was subsequently monitored by adenylate cyclase-mediated cAMP formation (Fig. 3b). Addition of the antagonist ($\pm$)propranolol reduced adenylate-cyclase activity to background. The agonist-dependent activation of G_s protein by the fusion protein was found to be in the same range as that for the receptor

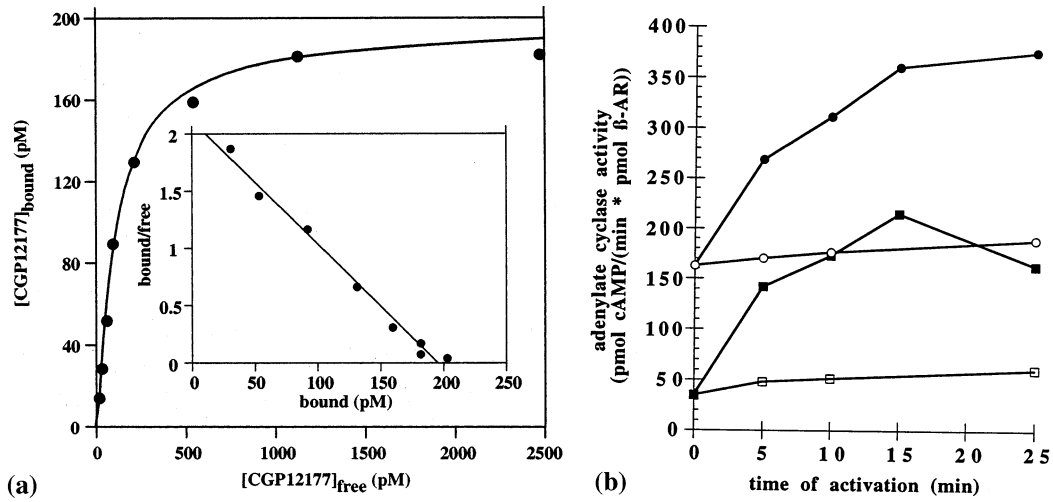

Fig. 3. Radioligand binding analysis and G-protein coupling of the optimized MalEβAR fusion protein produced in *E. coli*. (a) Saturation curve of [^{3}H]CGP-12177 binding determined on total bacterial membranes. The Scatchard transformation given in the inset indicates the presence of a single class of high affinity binding sites with a K_D-value of 96 ± 9 pM. The data presented are from one of at least two independent experiments with each point measured in triplicate. (b) Reconstitution of solubilized MalEβAR fusion protein and unfused receptor from *E. coli* (circles) and from stably transformed Sf9 insect cells (squares), respectively, with G_S protein purified from turkey erythrocyte. The receptor was activated in the presence of GTPγS for the indicated times with the agonist (−)isoproterenol (10 μM; filled symbols) which for basal determinations was replaced by the antagonist (±)propranolol (10 μM; open symbols). For each time point, activated G_S protein subsequently was assayed by addition of adenylate cyclase and formation of cAMP was measured as described earlier (Salomon et al., 1974).

alone which had been produced in stably transformed insect cells (Kleymann et al., 1993).

3.4. Purification attempts of the MalEβAR fusion protein produced in E. coli

Almost 90% of the binding sites were recovered after solubilization of *E. coli* membranes with the detergent dodecanoyl sucrose. In a first purification attempt of the MalEβARΔ132 fusion protein, crosslinked amylose was found which specifically binds MalE. However, this procedure proved to be extremely susceptible in respect of detergent concentration and, therefore, could not be used reproducibly with crude solubilizates. In order to provide an easy first purification step, a hexahistidine tag was engineered to the C-terminus of the MalEβARΔ132 fusion protein. The resulting MalEβARΔ132His6 fusion protein was purified by affinity chromatography on immobilized Ni^{2+} ions with a recovery of about 30%. As was revealed by immunoblot analysis, proteolytic fragments as well as a minor amount of full-length fusion protein were not retarded on the column (Fig. 4). However, some degradation products were still present in the eluate. Further purification of the pooled receptor-containing fractions on crosslinked amylose resulted in an almost homogenous protein preparation (Fig. 4) with a 60% recovery of binding sites leading to an overall yield of about 16%. The specific activity of the purified protein with 147 pmol mg^{-1} indicated that most of it was not capable of binding the radioactive ligand.

3.5. Characterization of the MalEβAR fusion protein produced in baculovirus-infected insect cells

In order to improve the production level of the fusion protein, we decided to use an eukaryotic expression system. There are several reports claiming high level production of adrenergic receptor in insect cells after infection with a respective recombinant baculovirus (George et al., 1989;

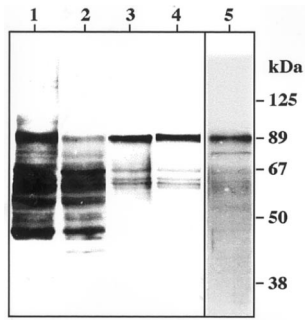

Fig. 4. Purification of the MalEβAR fusion protein from *E. coli*. Immunoblot analysis with anti-*Mal*E antibodies was performed using *E. coli* membrane solubilizate (1), the flow through (2), and the 100 mM imidazole eluate (3) of the Ni^{2+} charged Sepharose Fast Flow column. The 20 mM maltose eluate collected from the crosslinked amylose was analyzed by immunoblot analysis (4) and by silver staining (5). For better visualization of degradation products the immunoblots were over-developed.

Reiländer et al., 1991; Kobilka, 1995; Kwatra et al., 1995). Therefore, the construction encoding the MalEβARΔ132His6 fusion protein was adapted to this expression system. The coding region of the fusion protein was recloned into a baculovirus transfer vector and simultaneously the MalE signal peptide coding region was replaced by that of the honey bee prepromelittin (Fig. 5a). As expected, Sf9 insect cells which had been infected with the resulting recombinant baculovirus exhibited time-dependent production of

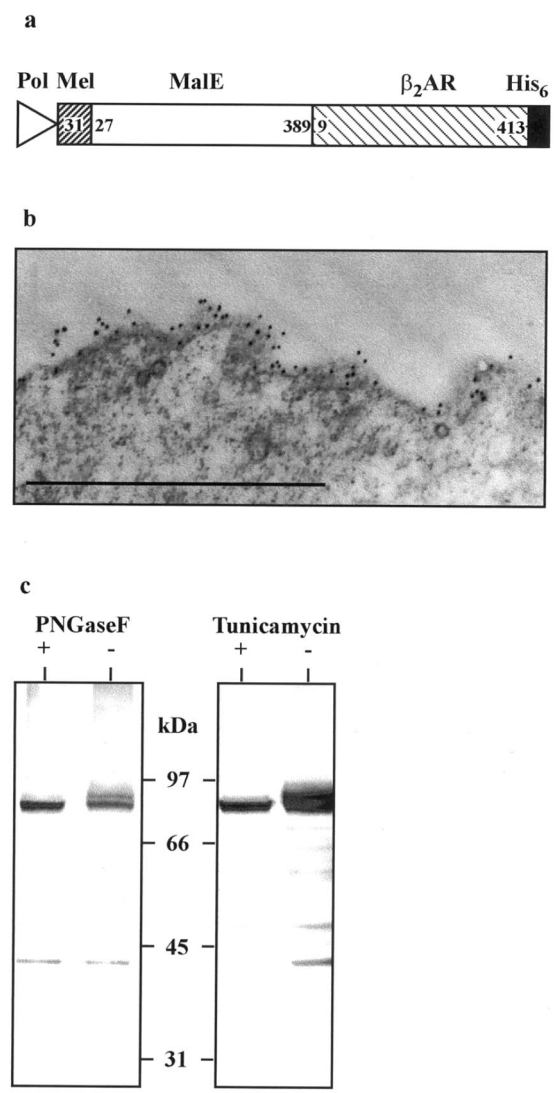

Fig. 5. Production of the MalEβAR fusion protein in baculovirus-infected Sf9 insect cells. (a) The fusion protein containing the signal peptide of the honey bee prepromelittin (Mel), for production in Sf9 insect cells was cloned under transcriptional control of the late baculovirus polyhedrin promotor (Pol). The construction otherwise was identical to that in plasmid pMalEβARΔ132His6. The number of amino acids derived from the signal-peptide containing vector pVL92Mel and the first and last amino acid of the fusion protein parts of the MalE and the β_2AR are given. His6, hexahistidine tag; MalE, *Mal*E gene without its own signal peptide; β_2AR, the human β_2AR fused to *Mal*E as indicated in Fig. 1b. (b) Pre-embedding immunogold labeling. Three days after infection, Sf9 insect cells were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS and later incubated with anti-*Mal*E antiserum. For demonstration of antiserum binding sites on the cell surface, the sample was incubated with goat anti rabbit-10 nm gold conjugate. For electron microscopic analysis thin sections were cut from the labeled and the resin embedded cells; bar = 1.8 μm. (c) Glycosylation analysis of the fusion protein produced in insect cells. Left panel: Membranes from baculovirus-infected Sf9 cells were treated with peptide *N*-glycosidase F (+) or mock-treated (−). Right panel: The glycosylation inhibitor tunicamycin (20 μg ml^{-1}) was supplemented to the growth medium of insect cells after infection with recombinant baculovirus (+). Infected cells which were grown in the absence of tunicamycin served as a control (−). In all cases visualization was performed by immunoblot analysis using the anti-MalE serum.

up to 4.2×10^6 binding sites per cell which after membrane preparation corresponded to about 17 pmol receptor fusion per mg membrane protein. As revealed by preembedding immunogold staining, the fusion protein was inserted into the plasma membrane of the infected insect cells, thus indicating correct folding and processing (Fig. 5b). No staining was observed in mock-infected cells (data not shown). Longer infection time than 3 days resulted in a higher production level as was detected by [^{3}H]CGP-12177 binding but immunoblot analysis showed severe proteolytic degradation of the fusion protein due to lysed insect cells (data not shown). As presented in Fig. 5c, in an immunoblot of membrane proteins prepared from insect cells 2 days after infection, mainly two bands in the range of about 89 kDa were recognized by the MalE antibody. After in vitro treatment of the membrane proteins with peptide *N*-glycosidase F, only the lower immunoreactive band was seen, indicating glycosylation of the fusion protein in the infected insect cells. Glycosylation of the recombinant fusion protein was further confirmed by treatment of infected insect cells with the glycosylation inhibitor tunicamycin which also resulted in disappearance of the upper immunostained band (Fig. 5c). As for the *E. coli* protein, saturation binding to the fusion protein from infected insect cells indicated a single high affinity binding site for [^{3}H]CGP-12177 ($K_D = 60 \pm 6$ pM) (Fig. 6a). Analysis of association ($k_{12} = 4.0 \pm 1.1 \times 10^6$ s^{-1} M^{-1}) and dissociation ($k_{21} = 50 \pm 5 \times 10^{-5}$ s^{-1}) kinetics resulted in a similar K_D-value of 125 ± 65 pM (data not shown). The typical order of affinities for the adrenergic ligands CGP-12177 ($K_D = 82 \pm 11$ pM), (−)propranolol (86 ± 20 pM), alprenolol (300 ± 60 pM), pindolol (1.4 ± 0.2 nM), (+)propranolol (13 ± 3 nM), albuterol (1.0 ± 0.2 μM), (−)isoproterenol (1.8 ± 0.4 μM), (±)epinephrine (20 ± 6 μM), (+)isoproterenol (28 ± 8 μM), and (−)norepinephrine (100 ± 18 μM) which were determined in competition binding experiments (data not shown) confirmed the functional integrity of the receptor. Competence in G-protein activation was assayed by coproducing the fusion protein with human heterotrimeric G_S protein ($G_{\alpha s\beta 1\gamma 2}$) after coinfection with respective recombinant baculoviruses as described previously (Grünewald et al., 1996). Membranes from

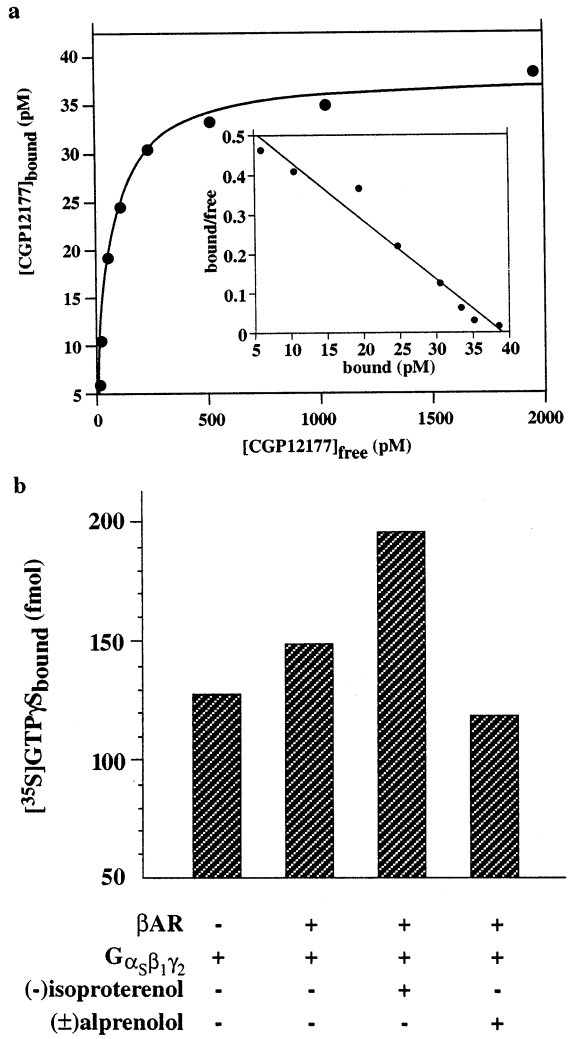

Fig. 6. Radioligand binding analysis and G-protein coupling of the MalEβAR fusion protein produced in baculovirus-infected Sf9 insect cells. (a) Saturation binding of [^{3}H]CGP-12177 to membranes prepared from infected Sf9 insect cells. A K_D of 60 ± 6 pM was calculated by non-linear regression. The Scatchard plot is given in the inset. The data presented are from one of at least two independent experiments with each point measured in triplicate. (b) Binding of GTPγS to phospholipid vesicles reconstituted from solubilized membranes of Sf9 insect cells infected with recombinant baculoviruses encoding the MalEβAR fusion protein (β_2AR) or the human stimulating G-protein subunits $G_{\alpha s}$, $G_{\beta 1}$, and $G\gamma_2$ ($G_{\alpha s\beta 1\gamma 2}$) in the absence or presence of the agonist (−)isoproterenol (100 μM) or the antagonist (±)alprenolol (1 mM).

infected insect cells were solubilized and proteins reconstituted into phospholipid vesicles. Activation of the G protein was assayed by addition of [^{35}S]GTPγS, a non-hydrolyzable GTP analog. [^{35}S]GTPγS binding was successively stimulated by the presence of recombinant receptor and the agonist (−)isoproterenol (Fig. 6b). In contrast, the antagonist alprenolol reduced activity below the level obtained in the absence of agonist.

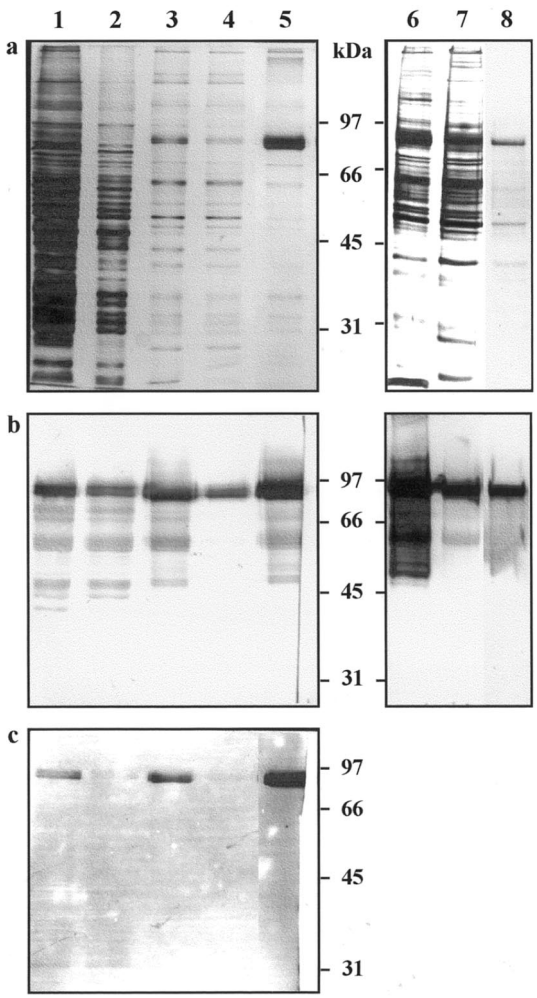

Fig. 7. Purification of the MalEβAR fusion protein from infected Sf9 insect cells. Purification of the fusion protein was carried out using immobilized Ni^{2+} affinity chromatography as a first step and either chromatography on crosslinked amylose or alprenolol sepharose as a second step. Solubilised membranes (1), flow through (2) and 100 mM imidazole eluate of the Ni^{2+} column (3) and the flow through (4) and the 20 mM maltose eluate (5) from the amylose chromatography analyzed by silver staining (a), and immunoblot analysis using either the anti-MalE serum (b) or the anti-hexahistidine tag antibody (c). In the right panels the result of a different purification attempt is shown. The eluate from the Ni^{2+} column (6) was applied to alprenolol affinity chromatography and analysed as described above (7, flow through; 8, 1 mM alprenolol eluate).

3.6. Purification attempts of the MalEβAR fusion protein produced in insect cells after infection with recombinant baculovirus

Solubilization of Sf9 membranes was performed with the detergent dodecanoyl sucrose at a concentration of 1%. The solubilized receptor fusion protein displayed a 10 times higher K_D for [^{3}H]CGP-12177 than the membrane-bound form as was found by saturation-binding experiments (830 ± 60 pM) as well as kinetic experiments (1160 ± 350 pM). In particular, the association of the ligand ($k_{12} = 6.1 \pm 0.6 \times 10^5$ s^{-1} M^{-1}) was slower whereas the dissociation was nearly unchanged ($k_{21} = 71 \pm 12 \times 10^{-5}$ s^{-1}). In a first step, the fusion protein was purified by affinity chromatography on immobilized Ni^{2+} ions. Subsequent purification by either amylose- or alprenolol-affinity chromatography yielded a homogenous preparation of the fusion protein as judged by silver staining or immunoblotting (Fig. 7). As already described above, also here about 15–30% of the binding sites were eluted with imidazole. A minor fraction of active receptor fusion was detected in the flow through which most probably was due to proteolysis of the C-terminal His-tag. More than 60% of the fusion protein applied to the column was not measurable afterwards but as judged by immunoblot analysis it was copurified with the intact fusion protein (Fig. 7b,c). Neither changing the pH, the ionic strength, the detergent, the protein and/or the detergent concentration nor changes in the conditions of the IMAC (e.g. exchange of the Ni^{2+} ions for Zn^{2+} ions; addition of dithiothreitol during the chromatography, elution using a pH gradient instead of imidazole) resulted in a higher recovery of functional fusion protein (data not shown). Unfortunately, further purification on crosslinked amylose led to higher losses in active

protein than during purification from *E. coli*. About 10% of the binding sites applied to the column were recovered in the eluate. Nevertheless, also here immunoblot analysis of the fractions indicated only a minor leakage of fusion protein into the run through fraction (Fig. 7b,c). In order to improve the purification yield as well as to remove denatured fusion protein, an alprenolol- instead of an amylose-affinity chromatography step was performed. Here, a large amount of fusion protein (Fig. 7b) was observed in the run through fraction which in respect to ligand binding proved to be inactive. Fusion protein which specifically could be eluted by alprenolol was pure as judged by silver staining and immunoblot analysis. However, the recovery of ligand-binding activity was about 5% as measured by filter binding assays and the specific activity was in the same range as reported for the purification from *E. coli*.

4. Discussion

In an attempt to enlarge the hydrophilic surface capable of intermolecular contact formation and to facilitate purification by affinity chromatography, the N-terminus of the hydrophobic human β_2AR was genetically fused to the hydrophilic MalE. For fast optimization of the linker region between the fusion partners which during heterologous production might be a target for host proteases, the fusion protein was produced in *E. coli*. As indicated by photoaffinity labeling, the fusion protein was proteolyzed in *E. coli*. Two bands with apparent molecular masses of about 89 and 45 kDa representing the fusion protein and the receptor alone, respectively, were labeled. Attempts to increase stability by the use of protease-deficient *E. coli* strains or by controlled deletions within the linker region failed. Finally, a random deletion approach using *Bal*31 yielded five constructs which exhibited improved stability against host proteases. Examination of the DNA sequence of these fusion proteins revealed no simple correlation with respect to amount or to location of the deletion and the stability, although, in the stable fusion proteins most or all of the linker region was deleted. One out of the five clones was selected for further characterization of the respective fusion protein. Previous to the study, six histidine residues (His6-tag) were attached to the C-terminus of the optimised fusion protein by genetic manipulation in order to allow subsequent purification by immobilised metal affinity chromatography (IMAC). The production level of this MalEβAR fusion protein in *E. coli* with maximally 6 pmol per mg membrane protein was significantly higher than that reported for a receptor β-galactosidase fusion protein (399 ± 75 fmol per mg membrane protein; Marullo et al., 1988) or LamB-receptor fusions (1.1 pmol per mg membrane protein, Chapot et al., 1990; 450 fmol per mg membrane protein, Marullo et al., 1989). The functional properties of the receptor itself were not altered by fusion to the MalE. Specific binding of [^{3}H]CGP-12177 as well as that of other adrenergic ligands to membranes prepared from recombinant *E. coli* was saturable. The affinities calculated from the data were in the range of that for the unfused receptor produced in eukaryotic expression systems (Reiländer et al., 1991; Kleymann et al., 1993). In accordance with results published for the other receptor fusion proteins produced in *E. coli* (Marullo et al., 1988; Freissmuth et al., 1991), coupling of the MalEβAR fusion protein to purified G_s-protein was also almost identical to that of the receptor alone. In conclusion, these data revealed that the receptor fusion protein heterologously produced in *E. coli* was resistant to action of host proteases and despite a missing glycosylation and palmitoylation fully functional with respect to ligand binding and G-protein coupling. Functional solubilization of the MalEβAR fusion protein was performed with the detergent dodecanoyl sucrose (Hekman et al., 1987). Dodecyl maltoside which also efficiently solubilized the fusion protein was excluded as it interacts with the MalE part of the fusion and, therefore, prevented binding of the protein to the amylose column. Sandwich purification of the MalEβAR fusion protein which resulted in an almost homogenous fusion protein preparation sufficient for biochemical and pharmacological analysis, was achieved by chromatography on immobilized Ni^{2+}-ions and subsequent chromatog-

raphy on an amylose column. However, the yield of fusion protein after elution from the Ni^{2+}-column with imidazol was low as was monitored by radioligand binding assays. The recovery of about 60% of fusion protein which was reached in the second purification step on the cross-linked amylose column was as high as expected. Since high concentrations of dodecanoyl sucrose prevented binding of the fusion protein to the amylose resin, this affinity chromatography was not employed as a first purification step. Nevertheless, the MalEβAR fusion protein purified from recombinant *E. coli* seemed to be at least 90% pure as judged by silver staining as well as immunoblot analysis.

To obtain larger amounts of receptor fusion protein, it was produced in Sf9 insect cells after infection with a recombinant baculovirus. In the baculovirus construction, the bacterial MalE signal sequence had been replaced by the honey bee prepromelittin signal sequence in order to circumvent problems in recognition and cleavage by the insect cell signal peptidase. It had already been reported that fusion of prepromelittin signal peptide to the β_2AR and the D_{2S} dopamine receptor enhanced insertion into the membrane and, therefore, enforced proper folding of the proteins (Guan et al., 1992; Gimpl et al., 1995; Grünewald et al., 1996). As expected, the production level in infected Sf9 insect cells with up to 17 pmol fusion protein mg^{-1} membrane protein was about five times higher than in *E. coli*. This value is in the range of those reported for the receptor alone produced in insect cells via the baculovirus expression system (George et al., 1989; Reiländer et al., 1991). The pharmacological properties of the fusion protein produced in the insect cells were as expected for an adrenergic receptor and agreed with results obtained for the receptor alone. The heterologously produced fusion protein was also capable of coupling to heterotrimeric G_s protein whose subunits were simultaneously coproduced in the insect cells by coinfection with different recombinant baculoviruses. Pre-embedding immunogold labelling revealed that the fusion protein was efficiently transported to the insect cells plasma membrane, indicating correct folding and processing. Contrary to the situation in *E. coli*, eukaryotic cells are capable of performing glycosylation of the recombinant protein. The N-terminus of the adrenergic receptor contains two consensus sequences for N-linked glycosylation (N-X-S/T) which after production of the receptor in infected insect cells were found to be glycosylated (Reiländer et al., 1991; Kobilka, 1995). During the optimization process of the MalEβAR fusion protein in *E. coli*, the first of the two N-terminal glycosylation sites had been deleted, still leaving the possibility of a once-glycosylated protein. Indeed, the fusion protein became glycosylated as two immunoreactive bands were detected in the immunoblots. The difference in apparent molecular mass of about 2–3 kDa which was detected after in vivo or in vitro deglycosylation of the fusion protein by tunicamycin or peptide N-glycosidase F, respectively, was in agreement with the assumption, that, in most cases, N-glycosylation of recombinant receptor produced in Sf9 insect cells is of the high-mannose type (Reiländer et al., 1991). Purification of the MalEβAR protein from infected Sf9 cells in a first trial was performed by the two-step affinity chromatography protocol. Although the C-terminus of the fusion protein was His6-tagged identical to that described by Kobilka (1995), we never obtained a recovery of about 87% after the IMAC as was stated in this previous report. Neither the use of high protein concentrations during this chromatography step nor the use of another receptor construct where the MalE in the fusion protein was exchanged against a c-*myc* epitope in an otherwise identical construction enhanced the recovery in our hands (data not shown). Despite the fact that the fusion protein was purified on crosslinked amylose, only a part of it was measurable by radioligand binding. Most likely, activity of the fusion protein was lost due to delipidation on the second column. Analysis of the fractions revealed that the fusion protein was almost homogenous after the two columns. Interestingly, also here the His6-tag was clipped off in part of the fusion protein population. Even sandwich purification of a receptor via attached tags cannot separate active from inactive receptor. This problem can be overcome by applying an affinity column which uses the internal binding properties

of the receptor in a final purification step. For this purpose, an alprenolol column has been used with yields of more than 50% of the active receptor (Kobilka, 1995; Kwatra et al., 1995). In our hands recovery from the alprenolol column was much lower. The low specific activity of the purified protein indicated that either the amount of purified active protein was underestimated by the filter-binding assay or that the fusion protein was recovered in an inactive form. Either the receptor was denatured or, more likely, delipidated and aggregated during elution from the column. Less likely, denatured fusion receptor unspecifically bound to the affinity column. Also, the MalE part of the fusion protein might be responsible for a decreased stability, therefore, inactivation during purification might occur at higher rates as for the receptor protein alone.

High-level production of G protein-coupled receptors for functional and structural studies is an important task. As milligram quantities of pure and homogenous protein will be required for crystallization attempts, it is worthwhile to first screen for the best expression system as well as for the best receptor construct. Proteolysis is a common problem during the production of a recombinant protein in a heterologous expression system. Therefore, successful attempts to reduce this problem will result in higher yields of the recombinant protein. Another problem is the purification of the recombinant protein. Here, the addition of tags or fusion to other proteins will enable subsequent immunological detection as well as purification. The choice of the fusion partner will also be important for subsequent crystallization attempts of the purified recombinant protein.

In this paper, we engineered a protease-resistant His-tagged MalEβAR fusion protein for heterologous production in *E. coli* and insect cells by using a random genetic approach. Purification of the fusion protein was achieved using the MalE fusion partner and the His6-tag. The optimized receptor fusion protein displayed pharmacological and biochemical properties virtually identical to that of the adrenergic receptor alone. The approach will be of general interest for the design of optimised fusion proteins for subsequent production in a suitable host.

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