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# Purification and functional characterization of the human $\beta_2$-adrenergic receptor produced in baculovirus-infected insect cells

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A human cDNA fragment bearing the complete coding region for the $\beta_2$-adrenergic receptor was introduced into the genome of *Autographa californica* nuclear polyhedrosis virus under the control of the polyhedrin promoter. Binding studies using [$^{125}$I]iodocyanopindolol showed that Sf9 insect cells infected with the recombinant virus expressed $\approx 1 \times 10^6$ $\beta_2$-adrenergic receptors on their cell surface. Photoaffinity labeling of whole cells and membranes revealed a molecular weight of ≈46000 for the expressed receptor. The receptor produced in insect cells is glycosylated but the extent and pattern differ from that of the receptor from human tissue. The heterologously expressed receptor was purified by alprenolol affinity chromatography, and was able to activate isolated Gs-protein.

$\beta$-adrenergic receptor; Baculovirus; Expression; Glycosylation; Affinity chromatography

## 1. INTRODUCTION

The $\beta_2$-adrenergic receptor is probably the best characterized hormone and neurotransmitter receptor. The receptor is coupled via a G-protein (Gs) to stimulation of the enzyme adenylate cyclase (for review see [1]). Recently the human $\beta_2$ receptor gene has been cloned and sequenced [2,3]. The cDNA contains an open reading frame encoding a protein of 413 aa ($M_r \approx$ 46 000). Hydrophobicity analysis of the human $\beta_2$AR reveals the presence of 7 transmembrane helices connected by hydrophilic loops. The hydrophobic core of the receptor is apparently involved in binding of the ligand to the receptor [4].

Detailed structural data are not available for any of the G-protein linked receptors. Determination of the structure will require milligram quantities of the receptors. We chose to express the human $\beta_2$AR utilising the baculovirus expression system to obtain the receptor protein in high yield (for review see [5,6]).

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*Abbreviations:* $\beta_2$AR, $\beta_2$-adrenergic receptor; CGP 12177, Ciba Geigy Product 12177; ConA, concanavalin A; Gs, stimulatory GTP binding protein; GTP[$\gamma$]S, guanosine 5'-*O*-(3-thiotriphosphate); [$^{125}$I]ICYP, [$^{125}$I]iodocyanopindolol; [$^{125}$I]ICYP-azide 2, [$^{125}$I]iodocyanopindolol-azide 2; MOI, multiplicity of infection; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Sf9-cells, Spodoptera frugiperda cells; WGA, wheat germ agglutinin

## 2. MATERIALS AND METHODS

### 2.1. *Cells and viruses*

The insect cell line Sf9 (*Spodoptera frugiperda*; ATCC accession number CRL 1711) was propagated at 27°C in TNM-FH-medium with 5% fetal calf serum and 50 mg/l gentamycin. Procedures for cell culture, viral infection and isolation of viral genomic DNA were carried out as described in detail by Summers and Smith [7]. The transfer vector pAC373 and the wild-type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) were kindly provided by Max Summers of the A&M college, Texas.

### 2.2. *Plasmid constructs*

Plasmid pTF3 (ATCC accession number 57537) was the source of $\beta_2$AR DNA [2]. To facilitate the construction of the recombinant transfer vector, the 2 kb *Eco*RI fragment of pTF3 was cloned into pBluescript (Stratagene). From the resulting plasmid pBS$\beta$2AR a 2 kb *Bam*HI/*Kpn*I fragment was isolated and ligated into pAc373 cut with *Bam*HI and *Kpn*I. To remove the complete 5' untranslated region preceeding the initiation codon of the $\beta_2$AR gene, pAc$\beta$2AR was digested with *Bam*HI and *Nco*I, and protruding ends were filled in with Klenow polymerase and deoxynucleotides. The 3' non-coding region of the $\beta_2$AR cDNA was not altered during construction [2]. The correctness of the construction was confirmed by restriction analysis and DNA-sequencing.

### 2.3. *Isolation of recombinant baculovirus*

Recombinant baculovirus was produced by co-transfecting Sf9 cells with 1 $\mu$g of genomic AcMNPV-DNA and 10 $\mu$g of plasmid pAc$\beta$2AR$\Delta$. Screening for recombinant virus was performed as described by Fung et al. [8] using radioactive transcripts of plasmid pBS$\beta$2AR. Putative recombinant virus was further analysed by two rounds of visual plaque screening and Southern hybridization of appropriately digested DNA from cells infected with recombinant virus. The total absence of any wild type AcMNPV was ensured by PCR using the culture supernatant of infected cells (Vasudevan, Reilländer, Maul and Michel, 1991, in preparation).

2.4. *Preparation of membranes, photoaffinity labeling and gel electrophoresis*

Membranes from Sf9-cells infected with recombinant baculovirus (MOI = 10) were prepared by nitrogen cavitation and differential centrifugation [9]. Purification of $\beta_2$AR from digitonin (1%, Serva, Heidelberg) solubilized membranes by affinity chromatography was done as described in [10]. Specific photoaffinity labeling of recombinant receptor with [$^{125}$I]ICYP-azide (2200 Ci/mmol, synthesized according to [11]) in whole cells and membranes prepared from infected cells was carried out as described previously [9,12]. As a control native human $\beta_2$AR from monolayers of $A_{431}E_3$ epidermoid cells was analyzed in parallel. SDS-PAGE (11% acrylamide and 0.8% bisacrylamide) was as described in [13] and the proteins were visualized by silver staining according to Oakley [14].

2.5. *Assays*

Glycosylation analysis of the recombinant human $\beta_2$AR by lectin affinity chromatography (Sigma) followed the protocol developed for analysis of the native human $\beta_2$AR [9]. Receptor numbers of intact cells and isolated membranes were determined by radioligand binding with [$^{125}$I]iodocyanopindolol (ICYP) as a ligand (2000 Ci/mmol, Amersham) [9]. Specificity of the assay was controlled by displacement with the hydrophilic $\beta$AR-specific ligand d,l-CGP 12177 (kindly provided by Dr. K.A. Jaeggi, Ciba-Geigy, Basel). Determination of protein content followed the method of Peterson [15]. Functional reconstitution of solubilized crude $\beta_2$AR and purified $\beta_2$AR with pure Gs isolated from turkey erythrocytes in lipid vesicles, and analysis of Gs stimulation with an adenylate cyclase preparation from rabbit myocardium was performed as described earlier [16–19]. Routinely, vesicles contained 2–8 fmol/$\mu$l of receptor and 9–20 fmol/$\mu$l of Gs. Concentration of cAMP was measured according to Salomon [20].

## 3. RESULTS AND DISCUSSION

Infection of Sf9-cells with recombinant baculovirus containing the human $\beta_2$-adrenergic receptor DNA resulted in a time dependent increase of $\beta_2$AR binding sites of whole cells as determined by radioligand assay (Fig. 1). 72–92 hours after infection of cells with recombinant baculovirus (MOI = 10) more than $10^6$ [$^{125}$I]ICYP binding sites/cell (equivalent to 12–17 pmol/mg membrane protein) were found. In comparison non-infected cells or cells infected with wild-type AcMNPV showed no significant binding of [$^{125}$I]ICYP. It is interesting that George et al. [21] obtained a similar concentration of binding sites in membrane preparations from baculovirus infected cells, whereas the number of receptors/cell differed considerably. The $K_d$ of 26 ± 6 pM for binding with [$^{125}$I]ICYP is in good accordance with results published earlier [9], and did not significantly change in the course of expression. The hydrophilic, membrane impermeable $\beta$AR specific ligand CGP 12177 which preferentially binds to receptors located on the cell surface [22] completely blocked [$^{125}$I]ICYP binding as well as incorporation of the $\beta$AR specific photoaffinity label [$^{125}$I]ICYP-azide 2.

Photoaffinity labeling of the receptor on whole insect cells with [$^{125}$I]ICYP-azide 2 followed by SDS-PAGE and autoradiography indicates a single polypeptide band with a $M_r$ of ≈ 46 000 (Fig. 2). Incorporation of the photolabel could be blocked stereospecifically by addition of the $\beta$AR specific ligands 1-isoproterenol

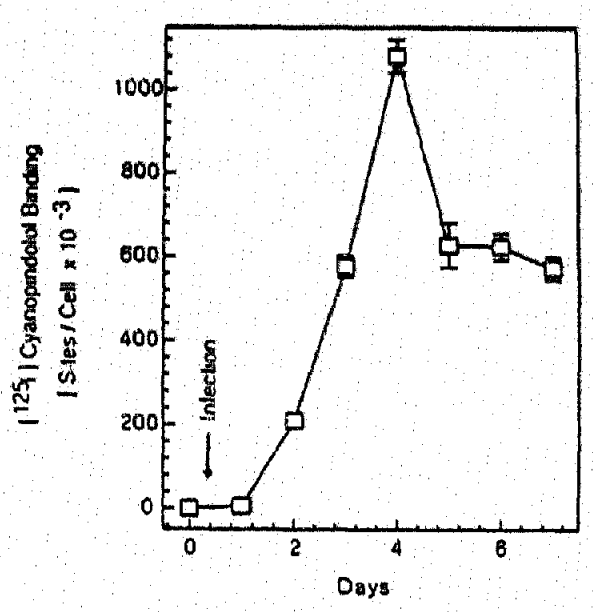


Fig. 1. Expression of human $\beta_2$AR in baculovirus-infected Sf9 cells. The cells were harvested immediately before (day 0), and at intervals of 24 h for 7 days after infection. The expression of $\beta_2$AR was determined by specific binding of [$^{125}$I]ICYP displaceable by d,l-CGP 12177 (1 $\mu$M). $B_{max}$ values were calculated from binding isotherms by computer-aided non-linear regression analysis [26] and are expressed as the mean ±SEM of triplicate measurements.

and CGP 12177 (Fig. 2). A band with the same $M_r$ was obtained when a membrane preparation of infected insect cells was used for photoaffinity labeling. The human $\beta_2$AR expressed in $A_{431}E_3$ cells which was analysed in parallel revealed a $M_r$ of ≈ 75 000 (Fig. 2). As the molecular weight of the $\beta_2$AR based on the gene sequence is ≈ 46 000, it appears that the receptor is

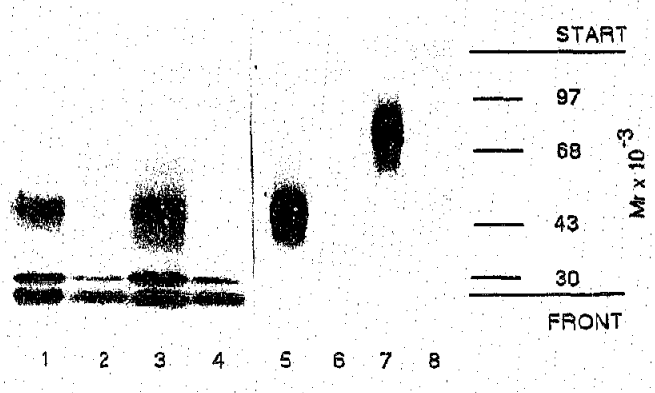


Fig. 2. Photoaffinity labeling of $\beta_2$AR expressed in $A_{431}E_3$ cells and in Sf9 cells infected with recombinant baculovirus. Membranes were prepared from Sf9 cells 3 days after infection. Sf9 cells in suspension (lanes 5 and 6) and membranes (lanes 1–4) were labeled with [$^{125}$I]ICYP-azide 2 as described in [12], and monolayers of $A_{431}E_3$ cells (lanes 7 and 8) were labeled as described in [9]. Specificity of [$^{125}$I]ICYP-azide 2 incorporation was checked by addition of 1 $\mu$M l-isoproterenol (lane 2), d-isoproterenol (lane 3), and d,l-CGP 12177 (lanes 4, 6 and 8).

highly glycosylated in $A_{431}E_1$ cells but is much less glycosylated in infected insect cells: $A_{431}E_1$ cells incubated with tunicamycin express the non-glycosylated form of the human $\beta_2$ AR with an apparent molecular weight of ≈ 40 000 estimated by photoaffinity labeling [9].

In order to study the glycosylation pattern of the $\beta_2$AR expressed in the insect cells, we applied lectin affinity chromatography as an analytical tool [23]. As shown in Fig. 3, [$^{125}$I]ICYP-azide 2 labeled receptor was bound to immobilized ConA with high affinity and therefore could be specifically eluted with α-methyl D-mannopyranoside. Proteins with N-linked carbohydrates of the high(oligo)-mannosidic type bind with high affinity, whereas certain bi-antennary complex type glycoproteins bind with low affinity to ConA. WGA which selectively interacts with N-linked glycoproteins of the complex type allows to differentiate between these two forms [23]. The $\beta_2$AR isolated from infected insect cells did not bind to immobilized WGA. These results indicate that in contrast to $\beta_2$AR isolated from human tissue which contains both types of N-glycans [9], the receptor from the insect cells probably carries only sugars of the high(oligo)-mannosidic type. This difference in the glycosylation pattern may explain the observed discrepancy in molecular weight between the receptor from $A_{431}E_1$ compared with that expressed in Sf9-cells [24,25].

The solubilized $\beta_2$AR from infected Sf9-cells was purified by alprenolol-sepharose affinity chromatography (Fig. 4). 150–200 pmol of solubilized receptor were bound per gram of alprenolol-sepharose. 10–12% of the bound receptor could be eluted with 1 mM l-alprenolol in an active form. As judged from silver-stained SDS-gels the eluted receptor was more than 90% pure (Fig. 4). In order to see whether the eluted receptor is functional after purification by affinity chromatography, we compared freshly solubilized and eluted receptors for their ability to couple and activate Gs-proteins. As presented in Fig. 5, stimulation of adenylate cyclase was nearly identical for both freshly solubilized receptor (2.8-fold above basal) and purified receptor (2.6-fold above basal). Pseudo first-order rates calculated by non-linear regression analysis of the data presented in Fig. 5 indicate that the stimulation was 3-fold slower for purified ($k_{on}$ = 0.06 ± 0.015 min$^{-1}$) than for freshly solubilized receptor ($k_{on}$ = 0.17 ± 0.05 min$^{-1}$). This difference in coupling efficiency may be attributed to the presence of tightly associated l-alprenolol which was used for the elution of the $\beta_2$AR from the affinity column.

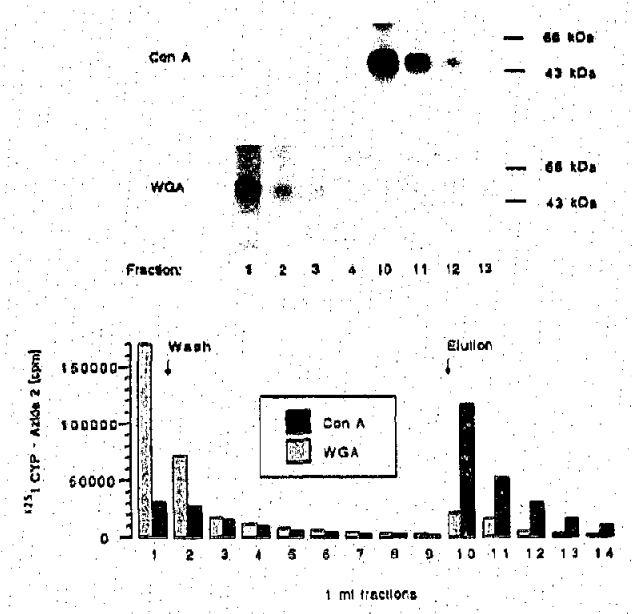


Fig. 3. Lectin affinity chromatography of $\beta_2$AR prepared from infected Sf9 cells. Membranes were prepared from Sf9 cells 3 days after infection. Receptors were photolabeled with [$^{125}$I]ICYP-azide 2 and subsequently solubilized in 0.5% Triton-X100. Soluble extracts were diluted and 1 ml aliquots were subjected to WGA- or ConA-Sepharose affinity chromatography (for details see [9]). After washing, columns were eluted with 0.5 M of the appropriate sugar (*N*-acetyl-D-glucosamine for WGA and α-methyl D-mannopyranoside for ConA). The sugars were dissolved in 50 mM borate buffer pH 8.0, 1 M NaCl. Flow-through-, wash- and eluted fractions (1 ml each) were counted in a Pharmacia-LKB gamma-counter. In all cases more than 95% of the applied radioactivity could be recovered. Aliquots of each fraction were applied to SDS-PAGE and visualized by autoradiography after washing (fraction 2–9), specific elution (fraction 10–14).

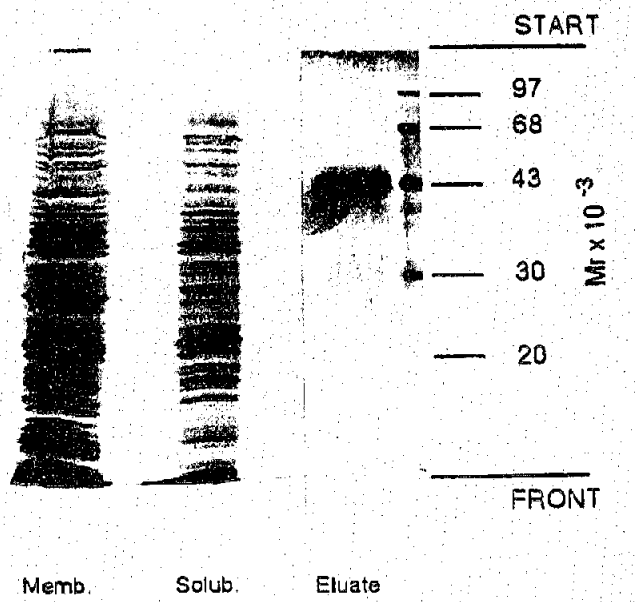


Fig. 4. Purification of $\beta_2$AR from infected Sf9 cells by alprenolol-Sepharose affinity chromatography. Membranes were prepared from infected insect cells, solubilized with 1% digitonin, and bound to the affinity resin. Elution was with 1 mM l-alprenolol (details see [10]). Aliquots of membranes (Memb.), solubilized membrane (Solub.), and eluate from the affinity column equivalent to 80 fmol of $\beta_2$AR were subjected to SDS-PAGE and visualized by silver staining (Eluate).

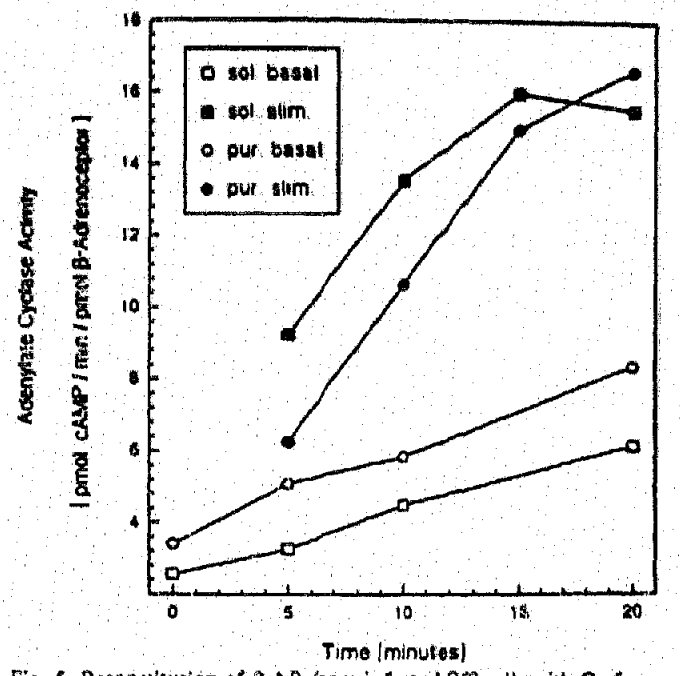


Fig. 5. Reconstitution of $\beta_2$AR from infected Sf9 cells with Gs from turkey erythrocyte. Crude (□, ■) or purified receptor (○,●) were mixed with purified Gs protein from turkey erythrocytes and a lipid mixture (phosphatidylethanolamin/phosphatidylserine/cholesterol hemisuccinate, 12:5:8) in the presence of 0.24% lauroyl sucrose. Vesiculation was accomplished by the Sephadex G-50 method [17]. The molar ratio of $\beta_2$AR to Gs after incorporation into vesicles was 1:2.5. l-Isoproterenol- (10 μM) dependent activation of Gs (●, ■) was carried out at 30°C in the presence of 60 nM GTP[γ]S. For basal determinations (○, □) l-isoproterenol was replaced by 10 μM d,l-propanolol. For each data point the amount of activated Gs was determined with a crude adenylate cyclase preparation as described [17].

Taken together, these results indicate that the human $\beta_2$AR expressed in the insect cells is biologically active and suitable for further biochemical, pharmacological and physical studies.

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