Communication

Enhancement of Membrane Insertion and Function in a Type IIb Membrane Protein following Introduction of a Cleavable Signal Peptide

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The human \( \beta_2 \) adrenergic receptor is a type IIb membrane protein. It has a putative seven-transmembrane topology but lacks an amino-terminal cleavable signal sequence. The mechanism by which the amino terminus of the \( \beta_2 \) receptor is translocated across the endoplasmic reticulum membrane is unknown. Furthermore, it is not known if translocation as a type IIIb protein is essential for the proper folding. Our studies indicate that conversion of \( \beta_2 \) receptor from a type IIb to a type IIIa membrane protein by introducing an NH\(_2\)-terminal cleavable signal sequence enhances translocation of the receptor into the endoplasmic reticulum membrane, thereby facilitating expression of functional receptor.

The \( \beta_2 \) adrenergic receptor is one of the most extensively characterized members of the family of G protein-coupled receptors, but little is known about its biosynthesis. Based on hydropathy analysis of the amino acid sequence and structural similarity to bacteriorhodopsin, it has been proposed that the seven hydrophobic segments of the \( \beta_2 \) adrenergic receptor are membrane-spanning domains, and that the amino terminus of the receptor is on the extracellular side and the carboxyl terminus is on the cytoplasmic side (Dixon et al., 1986). This model has been supported by biochemical and immunocytochemical studies (Dohlman et al., 1987; Wang et al., 1989).

Integral membrane proteins can be categorized into several different types, depending on the topological features such as the number of transmembrane segments and the orientation of the amino and carboxyl termini. The \( \beta_2 \) adrenergic receptor, and most G protein-coupled receptors, can be classified as type IIb proteins because they have a multiple membrane-spanning topology with their amino terminus on the extracellular side of the membrane but lack a cleavable signal sequence (Singer, 1990). A polytopic protein having a cleavable amino-terminal signal sequence is classified as type IIIa. Cleavable signal sequences are usually composed of about 20 amino acid residues with a basic polar amino terminus and an apolar core domain (von Heijne, 1983). Signal sequences serve to direct the insertion of proteins into the membrane cotranslationally by a mechanism involving the signal recognition particle (Singer, 1990; Walter et al., 1984). Once the protein is inserted, the signal peptide is cleaved from the mature protein at the carboxyl-terminal extent of the signal sequence by signal peptidase present in the lumen of the endoplasmic reticulum (ER).

The mechanism of translocation of the amino terminus and first membrane-spanning domain of type IIb proteins, such as the \( \beta_2 \) receptor, is poorly understood. It is believed that the membrane insertion of proteins without a cleavable signal sequence is assisted by internal signal sequences which are inserted in the membrane by the same mechanism that operates for cleavable signal sequences, except that no postinsertional proteolysis occurs (Blobel, 1982; Sabatini et al., 1982; Wickner and Lodish, 1985; Spiess and Lodish, 1986). The hydrophobic, putative membrane-spanning domains of the \( \beta_2 \) receptor are structurally most similar to known signal sequences. We sought to study the functional significance of the distinction between type IIIa and IIIb proteins, and to determine if a possible difference in the mechanism of translocation of a type IIb protein compared to a type IIIa protein might be important in the proper folding of G protein-coupled receptors. A cleavable signal sequence from influenza hemagglutinin was added to the amino terminus of the human \( \beta_2 \) adrenergic receptor. Receptors were expressed in insect cells and in a cell-free expression system. We observed that converting the \( \beta_2 \) receptor from a type IIb to a type IIIa protein enhances translocation of the receptor into the ER membrane and production of functional protein.

EXPERIMENTAL PROCEDURES

Construction of SF6b—The wild-type human \( \beta_2 \) receptor cDNA was cloned into the transcription vector pSP65 (Promega, Madison, WI). To create SF6b, a synthetic oligonucleotide of 76 base pairs was inserted in the Ncol site at the beginning of the \( \beta_2 \) receptor coding sequence (Fig. 1). This synthetic oligonucleotide encodes a modified influenza hemagglutinin signal sequence (Jou et al., 1986) and an antigenic epitope of 8 amino acid residues (the "Flag" epitope, IBI, New Haven, CT). The "Flag" epitope was inserted after the cleavage site for signal peptidase in order to verify the cleavage of the signal sequence (Fig. 1).

![Fig. 1. Diagram of the plasmid encoding SF6b. See "Experimental Procedures" for a description of the construction of pSP66SF6b.](http://www.jbc.org/)

1 The abbreviations used are: ER, endoplasmic reticulum; [H]DHA, [H]dihydroalprenolol; [3H]CYP, [3H]-cytopspin; PAGE, polyacrylamide gel electrophoresis; NYT, Asn-Tyr-Thr.

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sequence following its translocation. This epitope is recognized by a monoclonal antiseraum (M1 anti-Flag antibody, IIB) only if there is no amino acid sequence preceding it (Frickett et al., 1989). Thus, the M1 antibody binds to the receptor only if the signal sequence is cleaved.

RNA Synthesis—5'-Capped mRNA encoding β2 and SFβ2 were synthesized according to the method described previously (Kobilka, 1990).

In Vitro Translation—The β2 and SFβ2 receptors were synthesized in a cell-free translation system using rabbit reticulocyte lysate and microsomal membranes from Xenopus oocytes as previously described (Kobilka, 1990). Translation was carried out in the presence of [35S]methionine for 30 min, and then the reaction was chased by adding 1 mM unlabeled methionine for an additional 90 min. Ligand binding studies on the translated receptor were carried out as described below. Radiolabeled proteins were analyzed by electrophoresis on a 10% polyacrylamide gel (SDS-PAGE gel) (Laemmli, 1970). The radioactivity of protein bands on a SDS-PAGE gel was quantified by using an AMBIS β scanner (AMBIS, San Diego, CA).

Expression of β2 Receptors in Insect (Sf9) Cells—DNA sequences encoding the wild-type β2 receptor and SFβ2 were cloned into the baculovirus expression vector pVL1392 (provided by M. D. Summers, Texas A & M University). The calcium phosphate precipitation method was used to transfect plasmid and viral DNA into insect cells (Summers and Smith, 1987). The recombinant baculovirus stocks containing β2 or SFβ2 genes were isolated, plaque-purified, and titrated as previously described (Summers and Smith, 1987). Expression of the receptor was achieved by infecting Sf9 cells with various dilutions of the recombinant baculovirus stocks.

Radioiodination—[125I]Dihydroalprenolol ([125I]DHA) was used for receptor binding on whole cell preparations, and [35S]methionine ([35S]Me) was used for receptor binding on [35S]methionine-labeled protein. At the indicated time points after infection, Sf9 cells were harvested by centrifugation at 200 × g at 4°C. The cell pellets were resuspended in 1 ml of phosphate-buffered saline containing 10 nM [125I]DHA with or without other ligands. Reactions were incubated at 25°C for 1 h. For binding studies on in vitro translated receptors, aliquots (4 μl/binding tube) of translation reaction mixture were incubated in 500 μl of binding buffer with 1 mg/ml bovine serum albumin and 100 μM [3H]DHA for 90 min at 25°C. The binding assays were terminated by rapid filtration over GF/C glass fiber filters. Filters were washed twice with 4 ml of ice-cold buffer, and radioactivity was quantified in a liquid scintillation counter for [125I]DHA and [35S]S-labeled compounds or in a γ counter for [3H]DHA. Nonspecific binding was defined by including 10−6 M (-)alprenolol in the reaction. Protein concentration was determined by the method of Bradford (1976).

RESULTS

Expression of β2 and SFβ2 in Insect Cells—To determine the effect of the signal sequence on receptor expression in intact cells, DNA sequences encoding β2 and SFβ2 were inserted into the identical site of a baculovirus expression vector. Plasmid purified a Sf9 stocks of virus encoding each receptor were prepared, and the titer was determined. Cells were infected with four equivalent dilutions from equal titer virus stocks carrying either β2 or SFβ2 receptor genes. Receptor expression was assayed by [3H]DHA binding at 24, 48, and 96 h after infection. For each viral dilution and at each corresponding time point, the level of functional receptor produced (expressed as cpn specific binding/mg protein) was approximately 2-fold greater for SFβ2 compared with β2 receptors (Fig. 2 and Table I).

The pharmacological properties of SFβ2 receptors are indistinguishable from those of wild-type β2 receptors expressed in Sf9 cells. Competition studies were performed using two agonists (isoproterenol and epinephrine) and two antagonists (propranolol and alprenolol) against [3H]DHA binding. The curves for both β2 and SFβ2 are essentially identical (Fig. 3). The IC50 values (mean ± S.E. of three experiments) for β2 and SFβ2 receptors are 0.5 ± 0.1 versus 0.6 ± 0.1 μM for isoproterenol, 2.5 ± 1.1 versus 3.1 ± 1.6 μM for epinephrine, 1.6 ± 0.2 versus 2.3 ± 0.5 nm for alprenolol, and 1.2 ± 0.6 versus 1.7 ± 0.8 nm for propranolol.

Solubilized SFβ2 produced in insect cells bound efficiently to an M1 antibody column (data not shown). As indicated under “Experimental Procedures,” the Ca2+-dependent M1 antibody recognizes the Flag epitope only when nothing precedes the epitope at the amino terminus. Thus, the signal peptide is cleaved in insect cells.

Cell-free Expression of SFβ2—To explore the mechanism by which the signal peptide enhances the expression of functional receptor in insect cells, we studied expression of SFβ2 and wild-type β2 in a cell-free expression system composed of rabbit reticulocyte lysate and membranes prepared from Xenopus laevis oocytes. We have previously shown that receptor expressed in this cell-free translation system is capable of ligand binding and exhibits the same pharmacologic properties as receptors expressed in cultured cells (Kobilka, 1990). The mRNA encoding SFβ2 and wild-type β2 receptors was translated in the presence of [35S]methionine for 2 h at 30°C. The translation mixture was analyzed for ligand binding and incorporation of [35S]methionine-labeled protein into membranes. Maximal specific binding was observed in the presence of 10−8 M (-)alprenolol. Data are expressed as means of triplicates of a representative experiment. A total of three independent experiments were performed. See Table I for experimental variations.

TABLE I

Comparison of specific [3H]DHA binding of β2 and SFβ2 receptors expressed in Sf9 cells

| Virus dilution | Time (h) |
|---------------|----------|
| 1:10000       | 24       |
| 1:1000        | 48       |
| 1:100         | 72       |
| 1:10          | 96       |

| Dilution | Binding (cpm) |
|----------|---------------|
| 1:10000  | 2.3 ± 0.3     |
| 1:1000   | 2.3 ± 0.2     |
| 1:100    | 2.3 ± 0.4     |
| 1:10     | 2.3 ± 0.3     |

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brane protein than translation of \( \beta_2 \). The difference in expression between SF\( \beta_2 \) and \( \beta_2 \) in the cell-free expression system is therefore similar to the difference observed in the insect cell expression system.

To study the influence of the signal peptide on translocation of the receptor, the products of translation were analyzed by SDS-PAGE. Fig. 4 shows the result of translating mRNA encoding the \( \beta_2 \) receptor. Lanes 1 and 2 show that in the absence of the receptor mRNA, there is no significant production of \( ^{35} \)S-labeled protein. When \( \beta_2 \) mRNA is translated in the absence of microsomal membranes (Fig. 4, lane 3), the translated receptor protein migrates as a 42-kDa band on a SDS-PAGE gel. When membranes are added to the translation reaction, at least two additional bands with larger molecular mass (44 and 52 kDa) are apparent (Fig. 4, lanes 4 and 6). These slowly migrating species are receptor proteins that are modified by asparagine-linked glycosylation because they are not observed when translation is carried out in the presence of a competitive glycosylation inhibitor Asn-Tyr-Thr (NYT) (Lau et al., 1983) (Fig. 4, lane 5).

Fig. 5 shows the result of SDS-PAGE of SF\( \beta_2 \) and \( \beta_2 \) translated in the presence and absence of NYT. When mRNA encoding the \( \beta_2 \) receptor is translated in the absence of NYT, the fully glycosylated (52 kDa) receptor can be seen along with a considerable amount of radiolabeled species of smaller molecular mass (42 and 44 kDa), corresponding to the partially glycosylated or non-glycosylated receptor (Fig. 5, lanes 1-5) because of the different counting efficiencies. Two independent experiments were performed, and the individual values for each experiment are plotted as means of triplicates of a representative experiment. A total of three independent experiments were carried out, and the values obtained by liquid scintillation and AMBIS scanner are not directly comparable to the dried 10% polyacrylamide gel. Radioactivity for \( ^{125} \)I-CYP binding divided by cpm for \( ^{35} \)S-labeled membrane protein trapped on the filter for binding assays and counted in a Beckman liquid scintillation counter. The cpm values obtained by liquid scintillation and AMBIS scanner are not directly comparable because of the different counting efficiencies. Two independent experiments were carried out, and the individual values for each experiment are listed below.

![Fig. 3. Comparison of pharmacological properties of wild-type (A) and SF\( \beta_2 \) (B) receptors expressed in Sf9 cells. Sf9 cells were infected with baculovirus containing \( \delta_1 \) or SF\( \beta_2 \) receptor genes. Three days after the initial viral infection, cells were harvested, and radioligand binding was performed as described under "Experimental Procedures." \( ^{3} \)HIDHA (0.5 nM) was used for all binding assays, and the nonspecific binding was determined in the presence of 100 nM (-) alprenolol. Data are plotted as means of triplicates of a representative experiment. A total of three independent experiments were performed. EPI, epinephrine; ISO, isoproterenol; PROP, propranolol; ALP, alprenolol.

![Fig. 4. Autoradiogram of SDS-PAGE gel of \( \beta_2 \) receptors translated in the cell-free system. The receptor protein was synthesized in the presence of \( ^{35} \)Smethionine with or without microsomal membranes for 30 min at 25 \(^\circ\)C, and then chased with 1 mM unlabeled methionine for an additional 90 min. To inhibit glycosylation in selected translation reactions, 150 \( \mu \)g/ml NYT was included in the reaction mixture during the translation. The translation products were analyzed by SDS-PAGE as described under "Experimental Procedures." In lanes 1-5 the total translation mixture was analyzed, whereas lane 6 shows only membrane-associated proteins.

![Fig. 5. Effect of the amino-terminal cleavable signal sequence on membrane insertion of \( \beta_2 \) receptor. SF\( \beta_2 \) and wild-type \( \beta_2 \) receptors were synthesized in the presence of \( ^{35} \)Smethionine with or without NYT. At the end of translation, aliquots of 20-\( \mu \)l reactions were diluted in 100 \( \mu \)l of washing buffer (100 mM Tris, pH 7.4, and 5 mM EDTA), and centrifuged at 10,000 \( \times \) g for 15 min at 4 \(^\circ\)C. The membrane pellets were resuspended in another 100 \( \mu \)l of washing buffer and centrifuged again. The washed membrane pellets were then dissolved in sample buffer and analyzed by SDS-PAGE. Three independent experiments were performed with similar results.

| WT\( \beta_2 \) | SF\( \beta_2 \) | Ratio (SF\( \beta_2 \)/WT\( \beta_2 \)) |
| --- | --- | --- |
| A. Specific binding normalized to total membrane-associated \( ^{35} \)S-labeled protein |
| Experiment I | 0.05 | 0.22 | 4.3 |
| Experiment II | 0.06 | 0.12 | 2.0 |
| B. Specific binding normalized to fully glycosylated protein |
| Experiment I | 29.3 | 29.1 | 0.99 |
| Experiment II | 34.0 | 32.4 | 1.05 |

Table II

Comparison of the amount of functional receptors synthesized in vitro from mRNA encoding wild-type \( \beta_2 \) and SF\( \beta_2 \) receptors.
A Cleavable Signal Sequence Enhances β2 Receptor Expression

Fig. 6. Binding of detergent-solubilized β2 and SFβ2 to the M1 antibody column. [35S]Met-tide labeled β2 and SFβ2 were synthesized in the cell-free expression system and the membranes containing translocated protein were solubilized in 1% digitonin, 150 mM NaCl, 10 mM sodium phosphate buffer (pH 7.4), and 1 mM CaCl₂. Lane 1, solubilized SFβ2; lane 2, solubilized β2; lane 3, pass-through from binding of solubilized SFβ2 to M1 antibody column; lane 4, pass-through from binding of solubilized β2 to M1 antibody column; lane 5, SFβ2, eluted from the M1 antibody column in the presence of 5 mM EDTA; lane 6, β2, eluted from the M1 antibody column in the presence of 5 mM EDTA.

DISCUSSION

In the present study, we have examined the effect of converting the β2 receptor from a type IIIb membrane protein to a type IIIa membrane protein. The amino-terminal hydrophobic segment of a type IIIb protein is believed to serve as its own noncleavable signal sequence (Blobel, 1980; Singer, 1990). Of interest, cleavable sequences are believed to be inserted into the ER membrane with the amino terminus on the cytoplasmic side of the membrane. In contrast, the first membrane-spanning domain of G protein-coupled receptors is believed to be oriented with the amino terminus on the luminal side of the membrane. We sought to determine whether the addition of a cleavable signal sequence would alter receptor biosynthesis. Our results show that when the amount of functional protein (as assessed by ligand binding) is normalized to total membrane-associated protein ([35S]-labeled protein trapped by filtration through a glass fiber filter), translation of mRNA encoding SFβ2 produces approximately 2-fold more functional protein than the wild-type β2. This is comparable with the results obtained by expression of these receptors in insect cells (Fig. 2). The enhancement in expression of functional receptors that results from the addition of a signal peptide appears to be due to more efficient translocation of the modified receptor into the ER membrane. Fig. 5 shows that, in comparison to the wild type β2 receptor, a greater proportion of translated SFβ2 is fully glycosylated. While we have previously shown that the presence of N-linked oligosaccharides on the β2 receptor is not required for function, glycosylation is nevertheless a reliable indicator that the glycosylated domain has been translocated into the lumen of the ER. Our results indicate that the mechanism by which the amino terminus of the β2 receptor is translocated as a type IIIb membrane protein appears to be less efficient than the mechanism employed by type IIIa proteins. The increase in expression of functional protein afforded by the signal sequence would not be expected to have physiologic significance, yet it remains to be explained why this class of membrane proteins has evolved with a less efficient mechanism for translocation.

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