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BIOCHEMISTRY, BIOPHYSICS AND MOLECULAR BIOLOGY

Expression and Purification of an Engineered Human Endothelin Receptor B in a Monomeric Form

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Abstract—In humans, two endothelin receptors, ETa and ETb, are activated by three endogenous 21-mer cyclic peptides, ET-1, ET-2, and ET-3, which control various physiological processes, including vasoconstriction, vasodilation, and stimulation of cell proliferation. The first stage of this study it to produce a stable solubilized and purified receptor in a monodisperse state. This article is focused on the engineering, expression, purification, and characterization of the endothelin receptor B for subsequent structural and functional studies.

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Endothelin receptor B (ET_B) belongs to class A of the family of G protein-coupled receptors (GPCRs). Dysfunction of the endothelin system leads to many serious diseases such as hypertension, atherosclerosis, heart failure, kidney diseases, and various forms of cancer [1].

ET_B receptor is a subject of extensive research for over 25 years. During this time, its amino acid sequence and topology were determined [2], endogenous ligands that activate this receptor were identified, its expression and localization in the human body were studied, and its physiological role was investigated [3]. There are data obtained by site-directed mutagenesis regarding some functionally important amino acid residues activating intracellular cascades [1]. However, the structure of representatives of endothelin receptors is currently unknown, and therefore the location and shape of their ligand-binding pockets, the mechanisms of their activation, and their native oligomeric state remain unknown. In the absence of information on the receptor structure, drugs aimed at specific targets are usually selected using high-throughput screening. Over the past 25 years, more than a thousand antagonists affecting endothelin receptors have been developed. Nevertheless, the majority of them do not exhibit high specificity, selectivity, and efficacy and cause undesirable side effects on the human body due to ectopic activity primarily via one of the G proteins or β-arrestin [4].

High-resolution determination of the crystallographic structure of endothelin receptors in different functional states may provide an opportunity to study in detail the mechanisms of signal transduction into the cell and serve as a basis for rational search for new-generation drugs.

It is extremely difficult to crystallize receptors of the GPCR class mostly due to their high conformational dynamics. To date, successful structural studies have been performed for 30 of the 826 GPCR receptors belonging to classes A, B, C, and F. The crystalline structures of GPCRs have been used for finding drug prototypes by implementing the principles of rational design [5].

A stable, solubilized, and purified receptor in a monodisperse state is required as an initial step for crystallization and determination of its structure.

This article describes the first steps in this direction, aimed at further development of pharmaceutical

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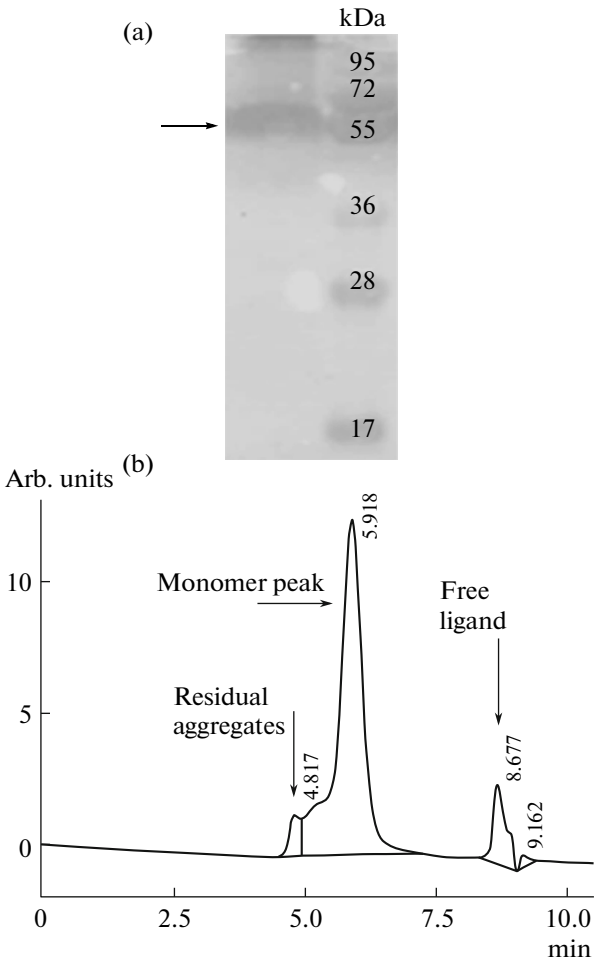

Fig. 1. Characteristics of the ET_B–BRIL preparation: (a) results of Western blot analysis, (b) results of analytical gel filtration.

drugs that regulate the function of endothelin receptors.

GPCR receptors exhibit a low stability, especially after their isolation from the native membrane and solubilization in detergent micelles. For this reason, to date only one GPCR, rhodopsin, has been crystallized in the native form [6], whereas all other receptors were subjected to various changes. To prepare ET_B preparation for structural studies, we used one of the most successful strategies consisting in creating a fusion protein by genetic engineering methods [7], expression of this construct in insect cells, solubilization in DDM/CHS micelles (*n*-dodecyl-β-*D*-maltoside/cholesteryl hemisuccinate, 5/1, wt/wt), and purification by metal chelate affinity chromatography [8]. The purity of the resulting protein preparation was tested by gel electrophoresis; monodispersity, by analytical gel filtration; and thermostability, by comparative analysis of denaturation curves.

Protein construct design. To increase the stability of the receptor and increase the chances of its crystallization, the gene for apo-cytochrome b562RIL (BRIL), a compact soluble protein [7], was inserted into the wild-type human ET_B gene (isoform A, UNIPROT database, P24530, http://www.uniprot.org/uniprot/P24530), with the substitution of amino acid residues SGMQIALNDHL in the third intracellular loop (ICL3). The insertion site was selected in accordance with the results of analysis of alignment with the A_{2A}AR-BRIL receptor sequence, the high-resolution structure of which is known [8]. Analysis of A_{2A}AR-BRIL and other GPCR structures with the ICL3-BRIL fusion shows the presence of a continuous α-helical transition between the transmembrane helix of the V-receptor and the N-terminal helix of the BRIL protein. To maintain the integrity of this α-helical transition, we selected a docking position in the V helix of the ET_B receptor, which was structurally equivalent to the docking position in A_{2A}AR-BRIL. In contrast, the docking site of the BRIL protein with helix VI has no exact α-helical transition and allows the presence of slight deviations. The unstructured flexible N and C termini were cut at positions ΔGln2–Arg64 and ΔPhe408–Ser440, respectively. Expression was performed using baculoviral expression system in the insect *Spodoptera frugiperda* cell line (Sf9), because this system is currently the most successful for the production of high quantities of functional GPCR receptors for structural studies [9].

Receptor solubilization and purification strategy. To purify membrane proteins, they must be solubilized from the native membrane environment with the use of detergents, some of which can destabilize the extracted protein or even change its conformation. Therefore, the selection of detergent for extraction and purification of membrane protein is extremely important for obtaining an adequate preparation for structural studies [10].

To isolate and purify ENDRB-BRIL, we decided to use the DDM/CHS detergent mixture (5/1 wt/wt), one of the most popular for work with GPCRs. This detergent mixture forms bicelle-like micelles with a disk-shaped bilayer, mimicking the membrane environment and stabilizing the embedded receptors [11]. CHS is a modified analogue of cholesterol, one of the major components of biological membranes. Cholesterol helps to maintain the bilayer rigidity and can directly bind to some GPCRs, playing an important role in their functioning [12].

Exposed cysteine residues on the receptor surface were blocked with iodoacetamide (Sigma, United States) prior to solubilization in order to prevent disulfide binding between receptors. To reduce protein aggregation at the expense of electrostatic forces, we added 10% glycerol and 300 mM NaCl to all buffers after solubilization.

One of the key factors for increasing the receptor stability is the selection of a ligand. Ligands can stabi-

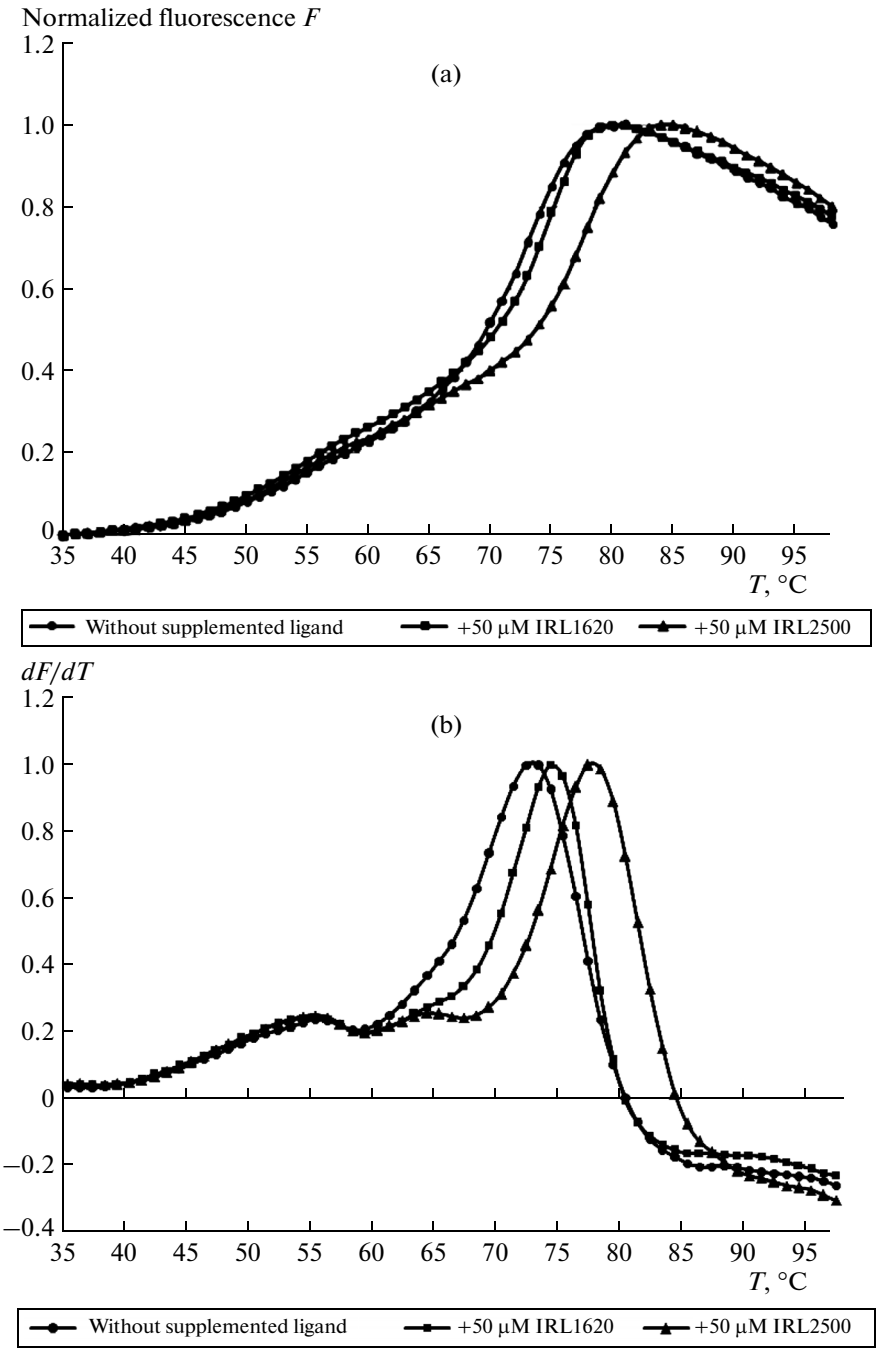

Fig. 2. Study of denaturation of the ET_B–BRIL preparation. (a) Denaturation curves of ET_B–BRIL. Here and in panel (b): ● without ligand, ■ in buffer supplemented with 50 μM IRL1620, and ▲ in buffer supplemented with 50 μM IRL2500. (b) Corresponding derivatives of the denaturation curves of the ET_B–BRIL preparation. Extremums correspond to the characteristic transition temperatures, T_M.

lize the receptor in a certain (e.g., active or inactive) state and reduce the overall dynamics of the protein [13]. It is not always possible to predict in advance which ligand will make it possible to obtain a high-resolution structure. The most important properties of a ligand are, apparently, the combination of the following conditions: high affinity (in a low nM or pM range), low dissociation constant (data are often not available due to the difficulty of measuring), high solubility (this is not a strict requirement but is relevant at least in the concentration range of 1–10 μM), optimum molecular weight (relatively large ligands often better stabilize the receptor, but if they are too flexible, this may be a disadvantage), and ligand type (antago-

nists are often preferable, although certain agonists can ensure sufficient stability for crystallization).

We decided to test two ligands—the low-molecular-weight antagonist IRL2500 (N-(3,5-dimethylbenzoyl)-N-methyl-(D)-(4-phenylphenyl)-alanyl-L-tryptophan, $K_i = 1.3$ nM, MW = 573.69 g mol^{-1} [3]), which was used as a ligand both in purification and thermal stability analysis, and the peptide agonist IRL1620 (suc-[Glu9, Ala11,15]-endotelin-1(8-21), $K_i = 0.016$ nM, MW = 1820 g mol^{-1} [3]), which was used only for the analysis of thermal stability. Both antagonist met the above requirements.

The ET_B–BRIL chimeric structure was expressed and purified in a complex with IRL2500 as described earlier for GPCR receptors [14] and then tested for homogeneity and thermal stability. Good monodispersity and high thermal stability are two most important characteristics of a protein suitable for further structural studies.

We performed several purification cycles. Usually, the protein purity was higher than 95%, and the analytical gel filtration (aSEC) profile showed primarily a single peak corresponding to the monomeric protein state (Fig. 1a). The number of dimers and aggregates was less than 5%. Western blots with anti-Penta-His antibodies (Qiagen N. V., the Netherlands; Fig. 1b) contained only one band corresponding to the expressed receptor and showed no sign of the protein degradation. The yield of the protein was estimated by the amplitude of the absorption peak at 280 nm during the passage through a gel filtration column using a calibration curve for BSA. The yield was 0.6 mg of the purified monomeric receptor per liter of expression medium.

Analysis of thermal stability. The protein was purified using 50 μM IRL2500. At the final step, the protein was concentrated to 2 mg/mL. The analysis of thermal stability using the CPM dye (7-diethylamino-3-(4'-maleimadylphenyl)-4-methylcoumarin, Life Technologies, United States) [15] was performed by diluting the protein sample 200 times with the reaction buffer without ligand as well as with buffers containing 50 mM IRL2500 or 50 mM IRL1620 (Fig. 2a). Protein denaturation curves were recorded with a Rotor-Gene Q mode real-time PCR instrument (Qiagen N. V., the Netherlands) at a temperature rise rate of 2 degrees/min (Fig. 2a). The corresponding normalized curves of derivatives are shown in Fig. 2b.

The low-molecular-weight antagonist IRL2500 stabilized the receptor better (dT ~ 6°C) than the peptide agonist IRL1620 (dT ~ 1°C). Therefore, the ET_B/IRL2500 complex has a better chance for successful crystallization.

In this study, we modified the gene for human ET_B by inserting a gene compact and stable protein BRIL (ET_B–BRIL) and expressed the resulting chimeric construct using the baculovirus expression system in insect cell lines *sf9*. The purification procedure was optimized for protein ET_B–BRIL with a purity of 95% and a high monodispersity. The typical yield of the purified monomeric protein was approximately 0.6 mg per liter of culture medium. The ligand-induced shift in the protein denaturation curve indicates that the solubilized and purified receptor is able to bind the ligand. Thus, the result of this study is the obtaining of a thermostable modified construct of human endothelin receptor B, which is suitable for further crystallization and structural studies.

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