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Increased expression of the integral membrane protein ErbB2 in Chinese hamster ovary cells expressing the anti-apoptotic gene Bcl-x_L

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ABSTRACT

Receptor tyrosine kinases (RTKs) are the second largest family of membrane receptors and play a key role in the regulation of vital cellular processes, such as control of cell growth, differentiation, metabolism, and migration. The production of whole-length RTKs in large quantities for biophysical or structural characterization, however, is a challenge. In this study, a cell engineering strategy using the anti-apoptotic Bcl-2 family protein, Bcl-x_L, was tested as a potential method for increasing stable expression levels of a recombinant RTK membrane protein in Chinese hamster ovary (CHO) cells. Wild-type and CHO cells stably overexpressing heterologous Bcl-x_L were transformed with the gene for a model RTK membrane protein, ErbB2, on a plasmid also containing the Zeocin resistance gene. While CHO cells exhibited a gradual decrease in expression with passaging, CHO-*bcl-x_L* cells offered an increased and sustained level of ErbB2 expression following continuous passaging over more than 33 days in culture. The increased ErbB2 expression in CHO-*bcl-x_L* cells was evident both in stable transfected pools and in clonal isolates, and demonstrated both in Western blot analysis and flow cytometry. Furthermore, the sustained high-level protein expression in CHO-*bcl-x_L* cells does not alter the correct membrane localization of the ErbB2 protein. Our results demonstrate that cellular engineering, specifically anti-apoptosis engineering, can provide increased and stable ErbB2 membrane protein expression in mammalian cells. This approach may also be useful for other membrane proteins in which large quantities are needed for biophysical and structural studies.

Introduction

The human proteome encodes nearly 1000 integral membrane proteins, representing nearly 30% of all proteins in the cell. These large numbers of membrane proteins are involved in a myriad of cell signaling pathways and processes within the cell [1,2]. Therefore, it is not surprising that membrane proteins represent nearly 50% of all potential pharmacological targets [3,4]. Understanding how these membrane proteins function is critical to the molecular medicine and biotechnology fields.

The second largest family of membrane receptors is the receptor tyrosine kinase (RTK) family. RTK-mediated signaling plays a key role in the regulation of vital cellular processes, such as control of cell growth, differentiation, metabolism, and migration [5]. Defects in RTK signaling lead to various developmental abnormalities and cancers, and the mechanism behind the pathologies is under intense investigation [6]. RTKs are single-pass membrane proteins with extracellular ligand-binding domains and intracellular kinase domains. They all signal via lateral dimerization in the membrane plane. Once a dimer is formed (usually in the presence of a ligand), the two cytoplasmic domains come into contact. The contact stimulates catalytic activity, and results in the intermolecular autophosphorylation of the receptor subunits. This activates the catalytic domains for the phosphorylation of cytoplasmic substrates and triggers signaling cascades [7].

Members of the RTK family have been classified based on their structural and ligand-affinity properties. One of the best studied subfamilies is the epidermal growth factor receptors (EGFRs or ErbBs), which has a demonstrated role in many human cancers [8,9]. Crystal structures of solved extracellular and catalytic domains of ErbB receptors have provided valuable insights into the process of ligand-induced dimerization [10–13]. Yet, many questions remain about the mechanism of receptor activation, and in particular about the transduction of the signal from the extracellular domains to the catalytic domains. The transmembrane domains have been shown to play a role in the process [14–16], and thus a complete understanding of the activation processes requires biochemical and crystallographic studies of whole-length receptors.

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The production of ErbB whole-length receptors in large quantities, however, is a challenge.

The main methods for determining the molecular structure of a protein, X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, require significant quantities of purified sample. While some stable, high abundance membrane proteins such as the G-protein-coupled-receptor (GPCR) rhodopsin have been purified and characterized from native tissues [17], most membrane proteins exhibit very low natural expression, thus prohibiting purification of sufficient amounts of protein from these sources. Therefore, researchers have looked at a variety of heterologous expression systems for the production of membrane proteins for structural and biophysical characterization.

Mammalian cells may be particularly useful for the expression of complex proteins due to their capacity to perform post-translational modifications and, thus, they are routinely used for production of large, recombinant biotherapeutics such as antibodies in commercial applications [18]. As a result, researchers have successfully expressed functional membrane proteins in baby hamster kidney (BHK) [19], human embryonic kidney (HEK 293) [19], COS-1 [20], and Chinese hamster ovary (CHO) [21,22] cells among others. Unfortunately, the expression level of recombinant membrane proteins from such systems is often lower than that of secreted proteins. Such difficulties in production may explain partly why membrane proteins account for a relatively small percentage of available protein structures.

A great effort has been made to increase the recombinant protein expression from mammalian cells, particularly in the area of secreted monoclonal antibodies. Cell engineering strategies, in particular, have targeted mammalian cell bottlenecks, including secretion [23], cell cycle [24], and apoptosis activation [25,26], all from within the cell. Anti-apoptosis cell engineering has the ability to maintain viability of cells in the stressful conditions of bioreactors, allowing for increased production run times and higher product yields. Expression of membrane proteins may represent one such stressful condition, especially since membrane protein overexpression has been shown to initiate the unfolded protein response [27]. As the need to understand the action and interactions of membrane proteins for medical and pharmacological efforts increases, the requirement for high-level membrane protein production systems becomes critical. Here, we examine the application of the anti-apoptosis gene *bcl-x_L* as a potential method for increasing stable expression levels of recombinant membrane proteins using ErbB2 as a model.

Materials and methods

Cell lines

Wild-type CHO and CHO-*bcl-x_L* cell lines have been described previously [28]. Cells were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), non-essential amino acids (Invitrogen), and L-glutamine (Invitrogen) in a humidified 5% CO_2 incubator at 37 °C.

DNA constructs

The plasmid pSV2_*neu*, encoding rat ErbB2 protein (Neu), was obtained from the laboratory of Prof. Daniel Donoghue (UC San Diego). The cDNA encoding the *erbB2* gene was excised from the pSV2_*neu* plasmid using the HindIII and SalI sites and inserted directionally into the pcDNA3.1/*zeo* vector (Invitrogen, Carlsbad, CA) using the HindIII and XhoI sites to generate pcDNA3.1/*zeo_erbB2*.

Transfections

Transfection of the vectors was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in OptiMEM reduced-serum medium (Invitrogen) according to the manufacturer's recommendations. For selection of CHO cells stably expressing the gene, Zeocin (Invitrogen) was added to a concentration of 0.250 mg/mL 24 h post-transfection.

Western blot analysis

Cells were lysed with lysis buffer containing 1% Triton X-100, 25 mM Tris–HCl, 1 mM EDTA, 150 mM NaCl, 1 mM Na_3VO_4, and complete-mini protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Cellular debris and insoluble proteins were removed by centrifugation, and the protein concentration of the cell extracts was measured using a BCA protein assay kit (Pierce, Rockford, IL). Equal total protein amounts of clarified cell lysates were denatured and loaded into 3–8% NuPAGE Tris–acetate gels (Invitrogen, Carlsbad, CA) and separated by electrophoresis at 150 V for 80 min. Separated proteins were then blotted onto nitrocellulose membranes (Biorad, Richmond, CA) at 75 V for 90 min. Membranes were blocked with 3% milk in Tris buffer saline (TBS) for 1 h. For detection of Bcl-x_L protein, blots were incubated with an anti-Bcl-$x_{S/L}$ antibody (clone S-18, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200. For detection of ErbB2 receptor, blots were incubated with a primary α-C-ErbB2 IgG antibody (Neu-C-18, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:500 in 3% milk/TBS overnight at 4 °C. A secondary anti-rabbit IgG HRP conjugate (w402B, Promega, Madison, WI) was used at a dilution of 1:2500, and incubated at room temperature for 90 min. Similarly, for detection of ErbB2 activation, blots were incubated with a primary α-phospho-ErbB2 antibody (2243P, Cell Signaling Technology, Beverly, MA). For detection of actin, blots were incubated with an anti-actin antibody (Sigma Chemicals, St. Louis, MO). Chemiluminescent detection of the HRP-conjugated antibody was achieved using the ECL Western blotting detection kit (GE Healthcare, Piscataway, NJ) and BioMax Light film (Kodak, Rochester, NY).

Immunofluorescence

Cells were transiently-transfected with pcDNA3.1/*zeo_erbB2*. Twenty-four hours after transfection, cells were fixed with 3% paraformaldehyde for 30 min at 4 °C, and blocked for 1 h at room temperature with 3% BSA in TBS. Cells were then incubated with the primary antibody, α-C-ErbB2 (Millipore, Billerica, MA) at a concentration of 1:500 in 1% BSA overnight. For fluorescence detection, an α-mouse IgG fluorescein conjugate (401214, Calbiochem, Los Angeles, CA) at a concentration of 1:100 in 1% BSA/TBS was used. Fluorescence imaging was carried out using a Nikon confocal microscope equipped with an imaging system.

Flow cytometry analysis

Cells were seeded in 6-well plates, incubated for 24 h, and resuspended in 5 mM EDTA. Resuspended cells were washed with 1% FBS in PBS, followed by incubation in a solution of α-c-ErbB2 (OP16, Calbiochem) in 1% FBS/PBS for 30 min. For fluorescence detection, an α-mouse IgG fluorescein conjugate (401214, Calbiochem) in 1% FBS/PBS was used. Cell staining was quantified using a FACSCalibur flow cytometer (Beckon Dickinson).