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Expression and purification of HER2 extracellular domain proteins in Schneider2 insect cells

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ABSTRACT

Overexpression of human epidermal growth factor receptor 2 (HER2/ErbB2/Neu) results in ligand independent activation of kinase signaling and is found in about 30% of human breast cancers, and is correlated with a more aggressive tumor phenotype. The HER2 extracellular domain (ECD) consists of four domains – I, II, III and IV. Although the role of each domain in the dimerization and activation of the receptor has been extensively studied, the role of domain IV (DIV) is not clearly understood yet. In our previous studies, we reported peptidomimetic molecules inhibit HER2:HER3 heterodimerization. In order to study the binding interactions of peptidomimetics with HER2 DIV in detail, properly folded recombinant HER2 protein in pure form is important. We have expressed and purified HER2 ECD and DIV proteins in the *Drosophila melanogaster* Schneider2 (S2) cell line. Using the commercial *Drosophila* expression system (DES), we transfected S2 cells with plasmids designed to direct the expression of secreted recombinant HER2 ECD and DIV proteins. The secreted proteins were purified from the conditioned medium by filtration, ultrafiltration, dialysis and nickel affinity chromatography techniques. The purified HER2 proteins were then analyzed using Western blot, mass spectrometry and circular dichroism (CD) spectroscopy.

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1. Introduction

Human epidermal growth factor receptor 2 (HER2/ErbB2/Neu) is involved in regulating cell growth, proliferation and survival. It is overexpressed in about 30% of human breast cancers and this expression is correlated with aggressive tumor phenotypes. This overexpression results in constitutive HER2 dimerization and signaling, typified by phosphorylation of HER2 cytoplasmic tyrosine residues [1–4]. Major clinical milestones in the treatment of cancers that overexpress HER2 are the FDA approval of trastuzumab and pertuzumab, humanized monoclonal antibodies that target HER2 [5–7]. Therefore, there is significant interest in the mechanisms of HER2 signal transduction and in strategies for disrupting HER2 signaling.

HER2 is a canonical receptor tyrosine kinase with several distinct functional motifs. HER2 consists of an extensive (~600 amino acid residues) extracellular domain; a hydrophobic, single pass transmembrane domain; a cytoplasmic tyrosine kinase domain; and a series of cytoplasmic tyrosine residues that serve as sites of phosphorylation. The HER2 extracellular domain (ECD) can be divided further into four functional domains (I, II, III, and IV) [8]. Domain IV (DIV) is near the transmembrane domain and stabilizes the protein–protein interaction between HER2 and its dimerization partner. Mutations in DIV impair phosphorylation [9] and signaling by heterodimers containing HER2. Nonetheless, the information regarding the role of the C-terminal region of DIV in heterodimerization is controversial [10]. Consequently, we have designed peptidomimetics that can bind to domain IV of HER2 and modulate HER2 signaling [11–16]. These peptidomimetics inhibit the protein–protein interaction of EGFR:HER2 and HER2:HER3 heterodimers [14]. In order to investigate the binding mode of such molecules on domain IV of HER2, structural elucidation of the HER2 extracellular domain–peptidomimetic complex is important.

Previous findings suggest that the ECD of EGFR family members can be expressed successfully in a number of host systems [17–22]. The aim of this project is to express and purify the HER2 ECD to study its binding to various antagonists. We used recombinant S2

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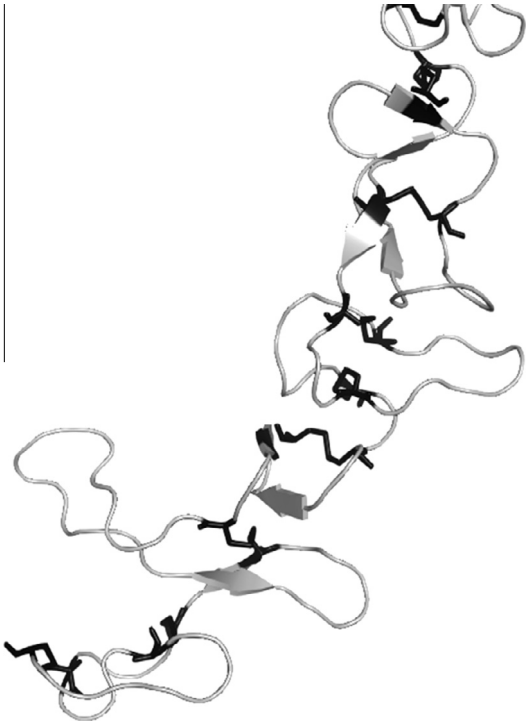

Fig. 1. Crystal structure of HER2 extracellular DIV [25]. Disulfide bonds are shown as dark sticks. Loops of DIV are stabilized the disulfide bonds and the structure of these loops in domain IV are structurally divided into seven modules (PDB ID: 3N85).

(Schneider2) cells for this purpose. S2 cells are derived from primary cultures of late stage embryonic cells of *Drosophila melanogaster* [23,24]. The rationale for using S2 cells for obtaining our protein is that these cells are easy to culture and grow as a semi-adherent monolayer at room temperature without CO_2. Moreover, S2 cells can be directed to secrete recombinant proteins into the culture medium in their native form, making for easy recovery and purification without using denaturing conditions. In contrast, recombinant proteins expressed in *Escherichia coli* are typically recovered following lysis of the *E. coli* under denaturing conditions, which necessitates refolding of the recombinant proteins following recovery. In addition, unlike recombinant proteins expressed in *E. coli*, recombinant proteins expressed in S2 cells will form intramolecular disulfide bonds. This is particularly important for the HER2 DIV, which contains intramolecular disulfide bridges and is structurally divided into seven modules [25–27] (Fig. 1) that are difficult to reconstitute from a recombinant protein expressed by *E. coli*. As a result of these advantages, S2 cells are being used for a variety of purposes, including the production of vaccine antigens and other heterologous proteins. It is noteworthy that the S2 system may allow for higher yields of these proteins than bacterial and yeast expression systems [28,29].

2. Materials and methods

2.1. Reagents

Drosophila Schneider2 (S2) cells, the *Drosophila* expression system-inducible/secreted kit, calcium phosphate transfection kit, hygromycin, fungizone, L-glutamine, probond nickel-chelating resin and purification columns were purchased from Life Technologies (Grand Island, NY). The Amicon 8400-stirred ultrafiltration apparatus was obtained from Millipore Corp. (Bellerica, MA). Fetal bovine serum (FBS) was obtained from ATCC (Manassas, VA). Schneider insect cell medium powder and copper(II) sulfate pentahydrate were from Sigma–Aldrich (St. Louis, MO). The pMT/BiP/V5-His B expression vector plasmid was from Invitrogen (Life Technologies) HER2 ECD and HER2 DIV genes were custom synthesized by Genscript (Piscataway, NJ). Dialysis cassettes were from Thermo Fisher Scientific (Rockford, IL). Penicillin/streptomycin was bought from Mediatech Inc. (Manassas, VA). Nalgene 0.2 μM rapid-flow bottle top filters were from VWR (Bridgeport, NJ). Antibodies for western (immunoblot) analysis were from Abcam, Inc. (Cambridge, MA) and Santa Cruz Biotechnology, Inc. (Dallas, TX). Novex® 4–20 % tris–glycine gels were obtained from Life Technologies (Grand Island, NY).

2.2. Maintenance of S2 cells

S2 cells were grown in Schneider2 insect medium with 10% heat inactivated FBS at 22 °C without CO_2. Initially, cells were grown at 28 °C. However, after transfection experiments, cell viability at 28 °C was low and at 22 °C cell viability of transfected cells was optimum hence, cell culture was carried out at 22 °C. When a density of 6–20 × 10^6 cells/mL was reached, they were subcultured at a 1:3 dilution as described by the supplier (Life Technologies).

2.3. Construction of expression vectors

Lyophilized pMT/BiP/V5-His A, B, C vectors were obtained from Life Technologies as a part of *Drosophila* expression system or (DES®) inducible/secreted kit. The complete extracellular domain of HER2 gene was subcloned into the pMT/BiP/V5-His vector using strategies described in earlier publications [17,22]. HER2 ECD/DIV was custom synthesized and subcloned it into the pUC57 plasmid by Genscript (Piscataway, NJ). Following amplification of the plasmid, the HER2 ECD/DIV gene was subcloned into pMT/BiP/V5-His vector to generate an expression construct suitable for expression in S2 cells.

2.4. Transfection

The calcium phosphate transfection kit was obtained from Life Technologies and the supplier's protocol was adopted for transfecting S2 insect cells. Cells were cotransfected with the pCoHygro selection vector and selected with hygromycin to establish stable cell lines. An expression vector to selection vector ratio of 19:1 (w/w) was used as per the supplier's protocol. Briefly, 35 mm plates were seeded with 3 × 10^6 cells in 3 mL medium and incubated at 22 °C. After 12 h, the cells had entered log phase growth and were used for transfection. Solution A of the transfection mix was prepared by mixing 2 M calcium chloride, the pMT/BiP/V5-His (expression) vector, and the selection vector in tissue culture sterile water. Transfection solution B was prepared from 2× HEPES buffered saline (50 mM HEPES, 1.5 mM Na_2HPO_4, 280 mM NaCl, pH 7.1). Solution A was added drop wise slowly to solution B with vigorous mixing and incubated at room temperature for 40 min to allow for the calcium phosphate–DNA precipitates to form. This mixture was added to cells and swirled to evenly distribute the precipitate. After incubating for 24 h, the cells were washed twice with S2 medium to remove the calcium phosphate precipitate. The cells were replated in the same plates in fresh medium and allowed to grow for two days. On day four, copper sulfate (500 μM final concentration) was added to the medium to induce the expression due to pMT (metallothionein promoter) for 24 h. The medium was replaced with medium containing hygromycin at 300 μg/mL. The selection was continued for four weeks to obtain stable transfectants. pMT/BiP/V5-His/GFP was used as a positive control.

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