Protein Expression and Purification 91 (2013) 54–60

Contents lists available at SciVerse ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Small structural differences of targeted anti-tumor toxins result in strong variation of protein expression

Roger Gilabert-Oriol [a], Mayank Thakur [a], Christoph Weise [b], Jens Dernedde [a], Benedicta von Mallinckrodt [a], Hendrik Fuchs [a], Alexander Weng [a,*]

[a] Institut Für Laboratoriumsmedizin, Klinische Chemie und Pathobiochemie, Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, Hindenburgdamm 30, D-12200 Berlin, Germany
[b] Institut Für Chemie und Biochemie, Freie Universität Berlin, Thielallee 63, D-14195 Berlin, Germany

ARTICLE INFO

Article history:
Received 6 June 2013
Available online 16 July 2013

Keywords:
Saporin
Dianthin
Protein expression
Protein structure
Targeted toxins
Tumor therapy

ABSTRACT

Targeted anti-tumor toxins consist of a toxic functional moiety that is chemically linked or recombinantly fused to a cell-directing ligand. Ribosome-inactivating proteins (RIPs), especially type I RIPs such as saporin or dianthin, are commonly used as toxin components. Although expression of type I RIP-based fusion proteins is well reported, the achievement of higher protein yields in heterologous expression systems through innovative strategies is of major interest. In the present study, the targeted toxins ^{his}saporin-EGF (SE) and ^{his}dianthin-EGF (DE) were expressed as fusion proteins under identical expression conditions. However, the total amount of DE was nearly two-times higher than SE. The identity of the heterologously expressed targeted toxins was confirmed by mass spectrometric studies. Their biological specific activity, monitored in real time, was almost equal. Sequence alignment shows 84% identity and a structural comparison revealed five major differences, two of which affect the secondary structure resulting in a loop (SE) to β-strand (DE) conversion and one introduces a gap in SE (after position 57). In conclusion, these structural variations resulted in different protein expression levels while codon usage and toxicity to bacteria were excluded as a cause. Minor structural differences identified in this study may be considered responsible for the protection of DE from bacterial proteases and therefore may serve as a lead to modify certain domains in type I RIP-based targeted toxins.

Introduction

Targeted toxins are a group of therapeutics used in tumor-specific therapies, a strategy that is based on the specific killing of cancer cells and the concomitant decrease of side effects in comparison to non-specific cancer therapies. Targeted toxins are composed of two functional moieties: a toxic enzyme, which induces cytotoxicity, and a targeting ligand, which directs the conjugate to the target cell [1].

Ribosome-inactivating proteins (RIPs)[1] are *N*-glycosylases (EC 3.2.2.22) that are prevalently used for the design of targeted toxins. In contrast to type II RIPs, type I RIPs lack a natural cell binding domain, which qualifies these enzymes as ideal candidates for targeted toxins. Type I RIPs include saporin from *Saponaria officinalis* L., dianthin from *Dianthus caryophyllus* L., gelonin from *Gelonium multiflorum* A. Juss., bouganin from *Bougainvillea spectabilis* Willd. and pokeweed antiviral protein from *Phytolacca americana* L. [2]. These enzymes mediate cleavage of a particular bond in the mammalian 28S RNA, resulting in the release of an adenine residue. Since this residue is required to bind eukaryotic elongation factors, the enzymatic modification results in the arrest of protein synthesis, which finally leads to apoptosis and cell death [3]. Typical targeting domains used as ligands for such toxins are growth factors, cytokines, antibodies or antibody fragments that recognize their respective receptors on cell surfaces [4].

The toxic moiety is coupled to the ligand by a chemical cross-linker, resulting in a protein conjugate. Alternatively, toxin and ligand are recombinantly fused at the DNA level by molecular cloning and expressed as a fusion protein [5]. Expression of fusion proteins comprising a type I RIP is a well reported method. An EGFR-targeted, dianthin-based fusion protein has been successfully expressed in bacteria [6] and saporin has been expressed as an EGFR-targeted fusion protein as well [7,8]. Saporin has also been fused to the basic fibroblast growth factor (FGF-2) [9], to the placental growth factor-2 (PlGF-2) [10] and to the amino-terminal fragment of human urokinase (ATF) [11]. However, new strategies to achieve higher protein amounts after heterologous expression are required for an optimized production of targeted anti-tumor toxins.

* Corresponding author. Fax: +49 30 8445 4152.
E-mail address: alexander.weng@charite.de (A. Weng).

[1] *Abbreviations used:* DE, ^{his}dianthin-EGF; EGF, epidermal growth factor; NCI, normalized cell index; NTA, nitrilotriacetic acid; RIP, ribosome-inactivating protein; SE, ^{his}saporin-EGF; EGFR, epidermal growth factor receptor.

1046-5928/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.pep.2013.07.004

In this study, the targeted toxins ^{his}saporin-EGF (SE) and ^{his}dianthin-EGF (DE) were expressed as fusion proteins and purified under identical conditions. Surprisingly, a significantly different expression level for these highly homologous proteins was observed, although their biological activity was comparable. It is hypothesized that minor but essential structural changes result in enhanced protein degradation by the expression host. Whether protein expression of additional members of the type I RIPs family follows the same pattern remains to be shown.

Materials and methods

Expression of ^{his}saporin-EGF and ^{his}dianthin-EGF

The plasmids ^{his}saporin-EGF-pET11d and ^{his}dianthin-EGF-pET11d coding for ^{his}saporin-EGF (SE) [12] and ^{his}dianthin-EGF (DE) [6] were transformed into *Escherichia coli* Rosetta™ 2(DE3) pLysS Competent Cells (Novagen, San Diego, CA, USA). To do so, either 100 ng of each was added to 20 μl bacteria. Rosetta™ 2(DE3) pLysS were transformed by a heat-shock (30 min on ice, 90 s at 42 °C and 1 min on ice). Further, 300 μL lysogeny broth (LB) medium without antibiotic was added to the bacteria and the suspension was incubated for 1 h at 37 °C while shaking at 200 rpm. A preheated LB medium-agar plate with 50 μg/mL ampicillin was inoculated with 100 μl bacteria suspension and the plate was incubated overnight at 37 °C.

A small amount (3 mL) of LB medium with 50 μg/mL ampicillin was inoculated with a colony from the plate and the bacteria were incubated for 8 h at 37 °C and 200 rpm. An amount of 50 μL of this bacterial suspension was added to 500 mL of LB medium with 50 μg/mL ampicillin and the culture was allowed to proliferate overnight at 37 °C and 200 rpm. Subsequently, the volume of bacterial suspension was scaled-up to a culture of 2.0 L and bacteria grew under the same conditions until an optical density ($A_{600\ nm}$) of 0.9 was reached. Thereafter, protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. Protein expression lasted for 3 h at 37 °C and 200 rpm. Finally, the bacterial suspension was centrifuged at 5000*g* and 4 °C for 5 min, re-suspended in 20 mL PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4 according to Dulbecco [13]) and stored at −20 °C until use.

To quantify the toxicity of targeted toxins on *E. coli* Rosetta™ 2(DE3) pLysS, 3 ml of LB medium supplemented with 50 μg/mL ampicillin was inoculated with a colony from the plate and the bacteria were incubated for 8 h as described before. Subsequently, 1 mL of this bacterial suspension was added to 100 mL LB medium supplemented with 50 μg/mL ampicillin and this time the culture was allowed to grow until an approximate optical density of 0.27. At that moment, protein expression was induced by adding IPTG at the final concentration of 1 mM and cell growth was monitored for the following 3 h. Optical density was read every 30 min with a SpectraMax 340PC Absorbance Microplate Reader (Molecular Devices, CA, USA). Statistical analyses were performed with IBM SPSS Statistics Version 21 (IBM Corporation).

Purification of ^{his}saporin-EGF and ^{his}dianthin-EGF

Bacterial suspensions were thawed and lysed with an ultrasound device (Branson Sonifier 250, G. Heinemann, Schwäbisch Gmünd, Germany). Lysates were centrifuged at 15,800*g* and 4 °C for 30 min and imidazole added to a final concentration of 20 mM. The fusion proteins were purified by Ni-nitrilotriacetic acid (NTA) agarose affinity chromatography (Protino® Ni–NTA agarose, Macherey–Nagel, Düren, Germany) by incubating the supernatant containing the expressed fusion proteins with 2 mL of Ni–NTA agarose under continuous shaking for 30 min at 4 °C in the presence of 20 mM imidazole. Subsequently, the material was poured into a 20-mL column. The column was washed three times with 10 mL wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole) and the fusion proteins were eluted by increasing concentrations of imidazole in wash buffer (31, 65, 125 and 250 mM). Elution was performed by 10 mL of each imidazole concentration and fractions of 2 mL each were collected. Eluate fractions were analyzed by SDS–PAGE [12% (w/v) gel], pooled afterwards and dialyzed overnight at 4 °C against 2.0 L PBS. Desalted fusion proteins were concentrated by an Amicon® Ultra-15 10,000 NMWL (Merck Millipore Ltd, Carrigtwohill, Ireland). A protein standard was prepared with bovine serum albumin (BSA) (Albumin Standard, Thermo Scientific, Rockford, IL, USA) and protein concentration was determined by a bicinchoninic acid assay (Pierce BCA Protein Assay, Thermo Scientific).

Mass spectrometric analysis

Peptides were obtained by trypsin in-gel digestion as described previously [14] and peptide masses were analyzed by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) using an Ultraflex-II TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a 200 Hz solid-state Smart beam™ laser. The mass spectrometer was operated in the positive reflector mode. Mass spectra were acquired over an *m/z* range of 600–4000. α-cyano-4-hydroxycinnamic acid (CHCA) was used as the matrix and protein digest samples were spotted using the dried droplet technique. MS/MS spectra of selected peptides were acquired in the LIFT mode [15]. Database searches were performed using Mascot (Matrix Science Ltd., http://www.matrixscience.com). One missed cleavage was allowed and mass tolerance was usually set at ±75 ppm.

Cell culture

HER14 cells (Swiss mouse embryo NIH-3T3 cells transfected with human EGFR, obtained from E.J. Zoelen, Department of Cell Biology, University of Nijmegen, The Netherlands) were grown in Dulbecco's modified Eagle's medium (DMEM) (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal bovine serum (FBS) (BioChrom KG, Berlin, Germany) and 1% Penicillin/Streptomycin (PS) (Gibco/Invitrogen, Karlsruhe, Germany) in a humidified 5% CO_2 incubator.

Real-time monitoring of cytotoxicity of fusion proteins

Cytotoxicity of SE and DE was analyzed in real time by the impedance-based xCELLigence System (Roche Applied Science, Mannheim, Germany). A 96-well e-plate (Roche Applied Science) that incorporates a sensor electrode array at the well bottom was pre-coated with 100 μL/well of 0.1% gelatin to facilitate the subsequent adhesion of cells. Gelatin solution was removed and firstly 50 μL DMEM supplemented with 10% FBS and 1% PS was pipetted in each well of the e-plate. The impedance was set to zero for medium alone. Thereafter 8000 HER14 cells were added (50 μL/well) and the cells were allowed to settle on the plate surface for 20 min. Following this, the plate was clamped onto the plate station and the impedance measurement was started. Cells were allowed to proliferate for 25 h as indicated by an increase of impedance. Twenty-five hours after seeding, the following compounds were added: 100 μL medium (untreated control), 100 μL medium supplemented with SE or DE (final concentration of 1 and 10 nM) or 100 μL medium supplemented with doxorubicin hydrochloride (Sigma–Aldrich, Steinheim, Germany) as toxin control at a final concentration of 1 and 1000 nM. Cells were