Journal of Immunological Methods 338 (2008) 1–6

Contents lists available at ScienceDirect

Journal of Immunological Methods

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

Research paper

Affinity-based entrapment of the HER2 receptor in the endoplasmic reticulum using an affibody molecule

Erik Vernet, Anna Konrad, Emma Lundberg, Per-Åke Nygren, Torbjörn Gräslund *

Department of Molecular Biotechnology, Royal Institute of Technology (KTH), Albanova University Center, Roslagstullsbacken 21, SE-106 91 Stockholm, Sweden

ARTICLE INFO

Article history:
Received 27 March 2008
Received in revised form 22 May 2008
Accepted 25 June 2008
Available online 29 July 2008

Keywords:
ErbB2
HER2
Affibody molecule
Endoplasmic reticulum
Flow cytometry

ABSTRACT

Interference with the export of cell surface receptors can be performed through co-expression of specific affinity molecules designed for entrapment in the endoplasmic reticulum during the export process. We describe the investigation of a small (6 kDa) non-immunoglobulin-based HER2 receptor binding affibody molecule ($Z_{HER2:00477}$), for use in affinity mediated entrapment of the HER2 receptor in the ER. Constructs encoding $Z_{HER2:00477}$ or a control affibody protein, with or without ER-retention peptide extensions (KDEL), were expressed in the HER2 over-expressing cell line SKOV-3. Intracellular expression of the full-length affibody constructs could be confirmed by probing cell extracts by Western blotting. Confocal immunofluorescence microscopy experiments showed extensive co-localization of the HER2 receptor and $Z_{HER2:00477}$-KDEL in the ER, whereas the use of a KDEL-extended control affibody molecule resulted in distinct and separate signals from cell surface-localized HER2 receptor and ER-localized affibody protein. This indicated a capability of the $Z_{HER2:00477}$-KDEL fusion protein to functionally interfere with the export process of HER2 receptor in a specific manner. Using flow cytometry and cell proliferation analyses, it could be shown that expression of the $Z_{HER2:00477}$-KDEL fusion construct in the SKOV-3 cell line resulted both in a marked reduction in cell surface level of HER2 receptors and that the cell population doubling time was significantly increased. Expression of the $Z_{HER2:00477}$-KDEL fusion protein in additional cell lines of different origin and with different expression levels of endogenous HER2 receptor compared to SKOV-3, also resulted in depletion of the cell surface levels of HER2 receptor. This indicated upon a general ability of the $Z_{HER2:00477}$-KDEL fusion protein to functionally interfere with the export process of HER2.

1. Introduction

Interference with the export of cell surface receptors can be performed through co-expression of specific affinity molecules designed for entrapment in the ER during the export process and is a promising tool for the study of their involvement in for example disease (Hu et al., 2005). For this purpose, ER-directed expression of fragments of receptor-specific antibodies (typically so called scFv antibody fragments) have been used, resulting in interaction between the antibody and the receptor in the ER and obstruction of the receptor's further movement in the secretory pathway. This strategy has been used to interfere with the export of different classes of proteins such as: oncogenic cell surface receptors, e.g. the epidermal growth factor receptor (Jannot et al., 1996); proteins essential for viral replication, for example the CCR5 co-receptor which is essential for cellular entry of several strains of HIV-1 virus (Steinberger et al., 2000); proteins capable of forming amyloid plaques, for example the β-amyloid precursor protein involved in Alzheimer's disease (Paganetti et al., 2005).

During recent years, several efforts to develop various classes of non-immunoglobulin affinity proteins have been

Abbreviations: ER, Endoplasmic reticulum; scFv, single-chain fragment; MLV, murine leukemia virus; t_d, doubling time.

* Corresponding author. Tel.: +46 8 55378332; fax: +46 8 5537841.
E-mail address: torbjorn@biotech.kth.se (T. Gräslund).

0022-1759/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jim.2008.06.005

described (Nygren and Skerra, 2004; Binz et al., 2005; Binz and Pluckthun, 2005). Such affinity proteins are typically developed via combinatorial amino acid variegation directed to a limited number of surface located positions in a so called scaffold protein, followed by affinity selection of variants capable of selective binding to a given target protein. Depending on the scaffold protein used (size, structure, SS-bridge content etc.) the potential for functional expression of resulting binders in different cellular compartments can be expected to differ.

One class of scaffold-derived affinity proteins are denoted affibody molecules, and are constructed by combinatorial engineering of a small (6 kDa), non-cysteine three-helix bundle protein Z, derived from staphylococcal protein A (Nord et al., 1995, 1997).

From libraries displayed on phages, affibody molecules have been isolated against numerous proteins of different character and origin including for example: IgA (Ronnmark et al., 2002), Factor VIII (Nord et al., 2001), CD28 (Sandstrom et al., 2003), gp120 (Wikman et al., 2006), amyloid-beta peptides (Gronwall et al., 2007) and the epidermal growth factor receptor (Friedman et al., 2007).

A target protein that has been extensively studied using affibody based reagents is the human HER2 receptor (Wikman et al., 2004; Ekerljung et al., 2006; Orlova et al., 2006; Lundberg et al., 2007; Tolmachev et al., 2007). This receptor is a 185 kDa glycoprotein, which can dimerize with other members of the epidermal growth factor receptor family, followed by sending e.g. proliferative signals (Graus-Porta et al., 1997). It is over-expressed in 25–30% of breast and ovarian cancers and over-expression is correlated with a higher rate of relapse and a poorer prognosis of affected patients (Slamon et al., 1987, 1989). Based on its accessibility on the cellular membrane and its relatively low expression on most non neoplastic cells, HER2 has been used as target for directed antibody-based immunotherapy by e.g. trastuzumab (Yeon and Pegram, 2005). In addition, various strategies based on intracellular antibodies (intrabodies) have been explored to prevent HER2 receptors from reaching the cellular membrane, and thereby prevent its signaling. In those studies, constructs encoding scFv antibody sub-fragments typically equipped with ER-retention peptides have been employed. Their expression have been able to prevent HER2 export to the cell membrane *in vitro*, resulting in a partial reversal of the transformed phenotype of HER2 expressing NIH/3T3 cells, manifesting itself as an inhibition of anchorage-independent growth in soft-agar (Beerli et al., 1994). Another group reported on a cytotoxic effect to SKOV-3 cells *in vitro* and also a reduction in the growth of SKOV-3 cells established as a tumor in athymic nude mice (Deshane et al., 1995). This last finding even lead to a phase I clinical trial where ovarian cancer patients were given the same construct, which however were unsuccessful in affecting the tumors (Alvarez et al., 2000).

High affinity affibody molecules have been developed to the HER2 receptor and have been characterized in a large number of studies, including *in vitro* analyses (Lundberg et al., 2007), *in vivo* imaging (Orlova et al., 2006) and *in vivo* therapy (Tolmachev et al., 2007). These studies have shown upon the capability of the affibody molecules to selectively recognize the mature and glycosylated HER2 target present on cell surfaces.

In this study, we have investigated the potential of one of the high affinity HER2 binding affibody variants for ER-entrapment of the HER2 receptor during the export process. Using a MLV-based gene delivery system, various affibody based gene constructs were delivered to the HER2 over-expressing cell line SKOV-3. The cellular localization of proteins, as well as the effects on HER2 surface expression and growth ability of cells were investigated. The ability of the HER2 binding affibody molecule to affect the HER2 surface level in additional cell lines with higher or lower basal expression of the receptor than SKOV-3, was also investigated.

2. Materials and methods

2.1. General

All DNA restriction and modifying enzymes were from Fermentas (Helsingborg, Sweden), New England Biolabs (In Vitro Sweden, Stockholm, Sweden) or Applied Biosystems (Foster City, CA, USA). The DNA sequence of all constructs was verified by cycle sequencing using an ABI Prism® 3700 Analyzer (Applied Biosystems). *Escherichia coli* strain RR1ΔM15 was used for all cloning work.

2.2. Plasmid construction, production and purification

All retrovirus-based affibody expression vectors were derivatives from pCMMP (Klein et al., 2000). The vector for ER targeted expression was constructed by inserting a linker into the multi-cloning site of pCMMP coding for the following: an N-terminal Ig κ-chain leader (amino acid sequence: METDTLLLWVLLLWVPGSTGD) for targeting the protein to the ER; two unique restriction sites (EcoRI and XhoI) for later sub-cloning of the affibody molecules; a HA-tag (hemagglutinin-tag) for detection (amino acid sequence: YPYDVPDYA); followed by the ER-retention signal (KDEL), resulting in the vector pMER-K. A control vector was also constructed that was identical to pMER-K except that the ER-retention signal was omitted. This vector was named pMER.

Affibody molecules Z_{Taq} and $Z_{HER2:00477}$ (Gunneriusson et al., 1999; Orlova et al., 2006) were sub-cloned into the EcoRI and XhoI sites of pMER-K and pMER.

2.3. Cell culture

The HEK-293T, RT4 and SKBR3 cell lines was obtained from ATCC. The SKOV-3, LS174T cell line was obtained from European Collection of Cell Cultures. All cell lines were cultivated as suggested by the manufacturers except: HEK-293T cells were cultivated in DMEM supplemented with 10% FBS, 1% non-essential amino acids and an Antibiotic–Antimycotic solution; SKOV-3 cells were grown in McCoy's 5A medium supplemented with 10% FBS and an Antibiotic–Antimycotic solution. All media and additives were from Invitrogen, Carlsbad, CA.

2.4. Production of retroviral particles and gene delivery to target cells

A Moloney's murine leukemia virus based system (Klein et al., 2000) was used for gene delivery of the affibody based