Biochimica et Biophysica Acta 1853 (2015) 1087–1095

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

# Selenoprotein W controls epidermal growth factor receptor surface expression, activation and degradation via receptor ubiquitination

Zeynep Alkan [a,*], Frank L. Duong [b], Wayne C. Hawkes [a]

[a] USDA-ARS Western Human Nutrition Research Center, 430 West Health Sciences Drive, University of California, Davis, CA 95616, USA
[b] Cedars Sinai Medical Center, Department of Medicine, 8750 Beverly Boulevard, Atrium 103, West Hollywood, CA 90048, USA

ARTICLE INFO

*Article history:*
Received 14 November 2014
Received in revised form 2 February 2015
Accepted 16 February 2015
Available online 23 February 2015

*Keywords:*
Selenium
Selenoprotein W
Epidermal growth factor receptor
Ubiquitination
Cancer

ABSTRACT

Epidermal growth factor (EGF) receptor (EGFR) is the founding member of the ErbB family of growth factor receptors that modulate a complex network of intracellular signaling pathways controlling growth, proliferation, differentiation, and motility. Selenoprotein W (SEPW1) is a highly conserved, diet-regulated 9 kDa thioredoxin-like protein required for normal cell cycle progression. We report here that SEPW1 is required for EGF-induced EGFR activation and that it functions by suppressing EGFR ubiquitination and receptor degradation. SEPW1 depletion inhibited EGF-dependent cell cycle entry in breast and prostate epithelial cells. In prostate cells, SEPW1 depletion decreased EGFR auto-phosphorylation, while SEPW1 overexpression increased EGFR auto-phosphorylation. SEPW1 depletion increased the rate of EGFR degradation, which decreased total and surface EGFR and suppressed EGF-dependent EGFR endocytosis, EGFR dimer formation, and activation of EGF-dependent pathways. EGFR ubiquitination was increased in SEPW1-depleted cells — in agreement with the increased rate of EGFR degradation, and suggests that SEPW1 suppresses EGFR ubiquitination. Ubiquitination-directed lysozomal degradation controls post-translational EGFR expression and is dysregulated in many cancers. Thus, suppression of EGFR ubiquitination by SEPW1 may be related to the putative increase in cancer risk associated with high selenium intakes. Knowledge of the mechanisms underlying SEPW1's regulation of EGFR ubiquitination may reveal new opportunities for nutritional cancer prevention or cancer drug development.

Published by Elsevier B.V.

## 1. Introduction

Epidermal growth factor (EGF) is a 53 amino acid polypeptide growth factor that regulates cell growth, proliferation and differentiation [1,2]. The EGF receptor (EGFR) is a receptor tyrosine kinase of the ErbB family, so-named because they are homologous with the erythroblastic leukemia viral oncogene. Four members of the oncogenic ErbB family have been identified: EGFR (ErbB1, HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4). EGFR was the first growth factor receptor to be identified in cancer cells and is considered a human proto-oncogene. EGFR is expressed in approximately one-third of cancers and the EGFR system is constitutively activated in many tumors. EGFR is activated by binding of various peptide growth factors, including EGF, transforming growth factor alpha (TGFα), amphiregulin, and heparin-binding EGF-like growth factor. EGFR ligands are produced by proteolytic cleavage from the extracellular domains of cell surface proteins or secreted precursor proteins. Most of the EGF family of growth factors are biologically active only in their soluble secreted forms, but some are also active as transmembrane precursor molecules [3]. Thus, EGFR is involved in endocrine, paracrine, and autocrine signaling mechanisms responding to systemic, local tissue, and intercellular cues. The EGFR network regulates growth, proliferation, differentiation and motility through pathways such as extracellular signal-regulated kinase (ERK), phosphatidylinositol-3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription [4]. The EGFR network crosstalks extensively with other receptor tyrosine kinases, G-protein coupled receptors, and cytokine receptors, and is a major hub in the extracellular signaling network [5–8].

Ligand binding leads to asymmetric EGFR dimerization, induction of the tyrosine kinase activity, auto-phosphorylation of the receptor, binding of cytoplasmic cofactors, internalization of the EGF-receptor complex, and initiation of a complex network of signaling cascades. Phosphorylated EGFR binds the E3 ubiquitin ligase Cbl, which adds ubiquitin at several lysine residues [9]. The ubiquitinated receptor is recognized by ubiquitin-binding domains in several members of the endosomal sorting complex for retrograde transport ("ESCRT") protein complex. Ubiquitinated EGFR is endocytosed and trafficked to lysozomes for degradation, or alternatively, EGFR can be de-ubiquitinated and recycled to the plasma membrane for re-use [10].

*Abbreviations:* ANOVA, analysis of variance; CHX, cycloheximide; $BS^3$, bis(sulfosuccinimidyl)suberate; SE, standard error of the mean; Sec, selenocysteine

* Corresponding author. Tel.: +1 530 752 4725.
*E-mail address:* zeynep.alkan@ars.usda.gov (Z. Alkan).

http://dx.doi.org/10.1016/j.bbamcr.2015.02.016
0167-4889/Published by Elsevier B.V.

The ubiquitination state of the receptor determines the relative rates of EGFR degradation and recycling, and ultimately, the amount of EGFR expressed on the cell's surface.

Selenoprotein W (SEPW1) is a 9 kDa thioredoxin-like protein with a selenocysteine (Sec) residue at the active site. SEPW1 is ubiquitously expressed and conserved in all major phyla except higher plants and fungi. SEPW1 is cell cycle regulated, is required for cell cycle progression, and is the only selenoprotein whose mRNA was increased by sub-micromolar concentrations of selenium in cultured human cells [11]. We found that G1-phase cell cycle arrest from SEPW1 depletion is mediated by phosphorylation of the p53 tumor suppressor protein via p38 MAPK and JNK2 under control of MKK4 [12–14]. We report here that SEPW1 is required for activation of EGFR upstream of MKK4 for the EGF-stimulated proliferation of prostate and breast epithelial cells. SEPW1 suppresses EGFR ubiquitination, which decreases lysozomal degradation and increases surface EGFR concentration and EGF responsiveness.

## 2. Materials and methods

### 2.1. Cell culture

RWPE-1 and MCF-10A cells were obtained from the American Type Culture Collection ("ATCC", Manassas, VA) and maintained in serum-free medium supplemented with EGF as described before [11].

### 2.2. siRNA transfections

Cells were transfected in medium containing either 0 or 5 ng/mL EGF per previously established protocols [14] with Silencer Select siRNAs targeting SEPW1 (s361, s363) or non-targeting control siRNAs #1 and #2 (ABI, Foster City, CA). Data identified as "SEPW1 siRNA" refers to siRNA s361, unless indicated otherwise.

### 2.3. SEPW1 overexpression vector transfections

Approximately 300,000 RWPE-1 cells per well were seeded in six well dishes on the day prior to transfection. The cells in each well were transiently transfected with either 2 μg SEPW1 *TrueClone* cDNA clone in a p-CMV6-Neo vector or the empty vector (Origene, Rockville, MD) with 10 μL Superfect Reagent (QIAGEN, Valencia, CA) per manufacturer's instructions. Three hours after the addition of transfection complexes, the cells were rinsed and supplied with fresh medium.

### 2.4. Cell cycle analysis

Propidium iodide staining of cellular DNA, flow cytometry, and data analysis were performed as described before [14].

### 2.5. Western blots

Western blot analyses were conducted using antibodies targeting the following proteins: EGFR (pan-specific), p-Tyr-992-EGFR, p-Tyr-1045-EGFR, p-Tyr-1068 EGFR, ubiquitin (Cell Signaling Technologies, Danvers, MA), β-actin, β-tubulin, and vinculin (Sigma). PVDF membranes were stripped with Restore Plus reagent (Pierce, Rockford, IL), and re-probed as needed. The immunoblots were imaged with a ChemiDoc XRS+ system (BioRad, Hercules, CA), and densitometry was performed with ImageLab software (BioRad). The chemiluminescence of each protein band was normalized to the average chemiluminescence of all the bands of the same protein from the corresponding immunoblot prior to combined statistical analysis from multiple blots.

### 2.6. Proteome profiler human phospho-MAPK arrays

Two hundred μg total protein from each lysate was hybridized to Proteome Profiler Human Phospho-MAPK antibody arrays (R&D Systems, Minneapolis, MN) per manufacturer's protocol, and all the arrays were imaged simultaneously with the ChemiDoc XRS + System. Densitometry was conducted with ImageLab software, and all the densitometry values were divided by 1000 for data presentation simplification. The average intensity of two negative control spots on each array was subtracted from the protein kinase spot intensities of the corresponding array for background correction.

### 2.7. Chemical crosslinking of cell surface receptors

EGF-starved cells were treated with 100 ng/mL EGF for 30 min at 4 °C, placed on ice, washed three times with ice-cold PBS, and then incubated with the non-membrane permeable crosslinking reagent bis(sulfosuccinimidyl)suberate ($BS^3$, Pierce), 3 mM, in PBS for 2 h at 4 °C. Excess $BS^3$ was quenched by addition of 1 M Tris–HCl, pH 7.4 to a final concentration of 20 mM and holding at 4 °C for 15 min. Cells were then washed with PBS, lysed, and the lysates analyzed by immunoblotting.

### 2.8. Fluorescent EGF internalization

Cells transfected and grown on coverslips were starved of EGF overnight and then stimulated with medium containing 970 ng/mL Alexa Fluor 488 streptavidin–biotin conjugated EGF (equivalent to 100 ng/mL EGF; Invitrogen), washed with PBS, fixed in ice-cold 100% methanol for 4 min, counter-stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma), and mounted on slides using SlowFade reagent (Invitrogen). The images were acquired on an FV1000 laser scanning confocal microscope (Olympus, Center Valley, PA). Total background-corrected Alexa Fluor 488 intensity of each image was measured with ImageJ software [43,44] and fluorescent EGF signal per cell was calculated by dividing the whole image signal intensity by the total number of cells in the image.

### 2.9. Measurement of surface receptor expression

Cell pellets were washed with ice-cold PBS containing 2 mM EDTA and 1% FBS (blocking buffer), fixed in PBS with 4% paraformaldehyde for 20 min on ice, washed again, and counted. Approximately 200,000 cells per sample were incubated with 1:50 EGFR antibody Ab-3 (Thermo Scientific) diluted in equal volumes of cold blocking buffer, washed, and then stained with 1:200 Alexa Fluor 488 conjugated anti-mouse antibody (Cell Signaling Technology). At least 40,000 events per sample were collected on a FACSCanto flow cytometer (BD Biosciences), and FITC area peak mean intensities were calculated by FACSDiva software (BD Biosciences).

### 2.10. Receptor immunoprecipitation

Cells were lysed with M-Per buffer containing HALT protease and phosphatase inhibitor cocktails (Pierce), and 400 μg total protein from each lysate were incubated with 1:100 EGFR antibody D38B1 (Cell Signaling Technology) in equal buffer volume for 1 h at 4 °C on a rocker. 50 μL Protein A/G Agarose Plus beads (Santa Cruz Biotechnology) were added to each sample prior to overnight incubation at 4 °C with rocking. The beads were washed five times with PBS and then boiled in reducing sample loading buffer for 5 min, and the supernatants were analyzed by immunoblotting.