Cellular Signalling xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Cellular Signalling

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

EGF stimulates the activation of EGF receptors and the selective activation of major signaling pathways during mitosis

Ping Wee, Huaiping Shi [1], Jennifer Jiang, Yuluan Wang, Zhixiang Wang *

Department of Medical Genetics, and Signal Transduction Research Group, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

ARTICLE INFO

Article history:
Received 11 September 2014
Received in revised form 10 November 2014
Accepted 24 November 2014
Available online xxxx

Keywords:
EGF receptor
Mitosis
Cell signaling
Cell cycle
ERK
AKT

ABSTRACT

Mitosis and epidermal growth factor (EGF) receptor (EGFR) are both targets for cancer therapy. The role of EGFR signaling in mitosis has been rarely studied and poorly understood. The limited studies indicate that the activation of EGFR and downstream signaling pathways is mostly inhibited during mitosis. However, we recently showed that EGFR is phosphorylated in response to EGF stimulation in mitosis. Here we studied EGF-induced EGFR activation and the activation of major signaling pathways downstream of EGFR during mitosis. We showed that EGFR was strongly activated by EGF during mitosis as all the five major tyrosine residues including Y992, Y1045, Y1068, Y1086, and Y1173 were phosphorylated to a level similar to that in the interphase. We further showed that the activated EGFR is able to selectively activate some downstream signaling pathways while avoiding others. Activated EGFR is able to activate PI3K and AKT2, but not AKT1, which may be responsible for the observed effects of EGF against nocodazole-induced cell death. Activated EGFR is also able to activate c-Src, c-Cbl and PLC-γ1 during mitosis. However, activated EGFR is unable to activate ERK1/2 and their downstream substrates RSK and Elk-1. While it activated Ras, EGFR failed to fully activate Raf-1 in mitosis due to the lack of phosphorylation at Y341 and the lack of dephosphorylation at pS259. We conclude that contrary to the dogma, EGFR is activated by EGF during mitosis. Moreover, EGFR-mediated cell signaling is regulated differently from the interphase to specifically serve the needs of the cell in mitosis.

1. Introduction

The epidermal growth factor (EGF) receptor (EGFR) is a 170 kD transmembrane glycoprotein consisting of a single polypeptide chain [1]. The binding of EGF at the cell surface induces dimerization of EGFR, which results in the activation of EGFR tyrosine kinase activity and receptor trans-autophosphorylation [2,3]. EGFR activation stimulates various signaling pathways leading to cell mitogenesis and survival [4,5]. EGFR is overexpressed or over-activated in many epithelial tumors and plays important roles in cancer development and progression [6].

EGFR plays important roles in initiating cell signaling to produce specific effects on cell growth and development [4,5]. The activated EGFR forms signaling complexes with many signaling proteins including Grb2, SHC, phospholipase C-γ1 (PLC-γ1), the p85α subunit of phosphoinositide 3-kinase (PI3K), Src, and Cbl [7–9]. The formation of the receptor-signaling protein complexes then initiates the activation of various signaling pathways. For example, the interaction between EGFR and SHC/Grb2 results in the recruitment of Sos to the plasma membrane to activate Ras. Activated Ras mediates Raf activation, which then phosphorylates and activates mitogen-activated protein kinase kinase (MEK), leading to the activation of extracellular signal-regulated kinases (ERKs). Activated ERK phosphorylates RSK that in turn translocates into the nucleus to activate transcription factors such as c-fos and SRF. Activated ERK may also translocate into the nucleus to activate transcription factors such as Elk1 and c-fos [7,9–14]. The activation of PLC-γ1 by EGFR regulates cell mitogenesis and migration [15–18]. The activation of PI3K by EGFR stimulates AKT/PKB activity, which protects the cell from undergoing apoptosis [19–21]. The activation of Cbl results in the ubiquitination and downregulation of EGFR [22–24].

However, all of the above knowledge regarding EGFR may only reflect its activation and function during the interphase, especially the G1 phase as these studies were conducted with the cells mostly in the G1 phase. The cell cycle is a series of events leading to cell replication. When plated at low cell densities in serum-containing medium, cultured cells start to proliferate, moving through the four phases of the cell cycle: G1, S, G2, and M. Progression through each phase and transition from one phase to the next is regulated by the coordinated action of kinases and proteases. When deprived of serum, cells continue

Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PLC-γ1, phospholipase C-γ1; PI3K, phosphoinositide 3-kinase; GAP, GTPase-activating protein; ERKs, extracellular signal-regulated kinases; MEK, mitogen-activated protein kinase kinase; I-phase, interphase; M-phase, mitosis; IP, immunoprecipitation; pY, phosphore tyrosine.

* Corresponding author. Tel.: +1 780 492 0710; fax: +1 780 492 1998.
E-mail address: zhixiang.wang@ualberta.ca (Z. Wang).
[1] Current Address: College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, China.

http://dx.doi.org/10.1016/j.cellsig.2014.11.030
0898-6568/

to cycle until they complete mitosis, whereupon they exit from the cell cycle into the G0 state. These cells can re-enter the G1 phase of the cell cycle by the addition of serum or growth factors [25,26]. Growth factors, including EGF, regulate cell cycle progression, especially the G0 to G1 transition and G1-S progression. Growth factors must be present until the restriction point (R point) in the G1 phase to stimulate entry into the cell cycle and proliferation. After the R point, growth factors are not required to complete the other stages of the cell cycle [25]. We and others also showed that two pulses (30 min) of growth factors including platelet-derived growth factor and EGF, separated by 8 h, are also able to drive the cell cycle [27,28]. While it has been proposed that the G0 to S interval is the only portion of the cell cycle that is regulated by growth factors [25], there have been sporadic publications showing a minor role for growth factor-mediated cell signaling in S and G2 phase [29–35].

The role of growth factor-induced cell signaling in mitosis has been rarely and poorly studied. Mitosis (M phase) is the most dynamic period of the cell cycle, involving a major reorganization of virtually all cell components. A hallmark of cancer involves the cancer cell's ability to sustain chronic proliferation [36]. A major difference between cancer cells and normal cells is that the cancer cells are much more mitogenic and show a higher frequency of mitosis. Therefore, most cancer drugs are designed to specifically target mitotic cells [37]. It has been reported that while there is no change in the number of surface-exposed EGFR between the interphase and M phase of the cell cycle, EGF-induced activation of EGFR and downstream signaling is tightly suppressed in the M phase due to a decrease in ligand binding affinity and the inability of EGF to induce receptor dimerization [38]. It has also been shown that EGFR, ERK2, GTPase-activating protein (GAP) and PLC-γ1 are less phosphorylated in M-arrested cells than they are in the interphase [39]. A further research showed that Cdc2 inhibits EGFR-stimulated ERK activation during mitosis by primarily targeting signaling proteins that are upstream of MEK1 including EGFR [40]. These studies suggest that inhibition of EGFR activity and its downstream signaling pathways underlie the importance of keeping the cell sheltered from extracellular signals when it undergoes division, and is beneficial for preventing gene expression so to preserve the energy needs that are required for mitotic structural changes [38–40].

However, in a recent research to study EGF-induced EGFR endocytosis during the M phase, we have shown that at the M phase, EGFR is expressed at the same level as in the interphase and is also activated by EGF to the same level as in the interphase as shown by pY992 [44]. Here we further studied EGF-induced activation of EGFR and the major downstream signaling pathways during cell mitosis. We showed that EGFR was strongly activated by EGF during mitosis as all the five major tyrosine residues including Y992, Y1045, Y1068, Y1086, and Y1173 were phosphorylated to a level similar to that in the interphase. We further showed that the activated EGFR selectively activated some downstream signaling pathways while avoiding others. Activation of EGFR resulted in the activation of AKT2, but not AKT1, which may be responsible for the observed effects of EGF against nocodazole-induced cell death. Activated EGFR also activated c-Cbl and PLC-γ1, two signaling protein with multiple cellular functions, during the M phase. However, activated EGFR is unable to activate ERK1/2 and their downstream substrates RSK and Elk-1. While it activated Ras, EGFR failed to fully activate Raf-1 in mitosis due to the lack of phosphorylation at Y341 and the lack of dephosphorylation at pS259.

2. Materials and methods

2.1. Antibodies and chemicals

Mouse monoclonal anti-PLC-γ1 antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). All of rest primary antibodies were from Santa Cruz Biotech (Santa Cruz, CA). The horseradish peroxidase (HRP)-conjugated secondary antibodies were from Bio-Rad (Hercules, CA) and the redeeming-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). The cell culture reagents were from Invitrogen (Carlsbad, CA). U Mammalian Protein Extraction Reagent (M-Per) was purchased from Thermo Fisher Scientific Inc. (Rockford, IL USA). Vybrant MTT Cell Proliferation Assay Kit was from Invitrogen (Grand Island, NY). Unless otherwise specified, all other chemicals were from Sigma (St. Louis, MO).

2.2. Cell culture and treatment

The cell lines that were used include HeLa cell line stably expressing H2B–GFP (HeLa H2B–GFP), Cos7 fibroblasts and CHO cell lines stably expressing a YFP-tagged, wild-type EGFR (CHO-EGFR) [41]. The HeLa H2B stable cell line is a generous gift from Dr. Wahl (The Salk Institute for Biological Studies). It has been shown that the H2B–GFP fusion protein is incorporated into nucleosomes without affecting cell cycle [42].

All cells were grown at 37 °C in Dulbecco's modified Eagle's medium containing 10% FBS and were maintained in a 5% CO2 atmosphere. For CHO cell lines, G418 was added to a final concentration of 500 μg/ml. For the HeLa H2B–GFP cell line, blasticidine was added as a supplement to a concentration of 2 μg/ml to maintain the trans-gene expression. EGF was used at a final concentration of 1 to 50 ng/ml. To inhibit EGFR kinase activity, cells were incubated with AG1478 at 1 μM for 30 min.

To collect lysates for mitotic cells, cells were arrested in prometaphase by treating with nocodazole (200 ng/ml) for 16 h. The nocodazole-arrested cells were treated with EGF (50 ng/ml, except indicated otherwise) for 5, 15, 30 min, 1 or 2 h. Mitotic cells in serum-free media were dislodged by gently tapping the plates on ice. The cells were washed with PBS and centrifuged at 1000 rpm. The obtained mitotic cells were then lysed with M-Per in the presence of phosphatase and protease inhibitors including 100 mm NaF, 5 mM MgCl2, 0.5 mM Na3VO4, 0.02% NaN3, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml aprotinin, and 1 μM pepstatin A. To collect lysates for interphase cells, cells were serum starved for 16 h and then treated with EGF for 5, 15, 30 min, 1 or 2 h. The cells were collected by scraping on ice in cold M-per buffer in the presence of phosphatase and protease inhibitors.

2.3. Immunoblotting

Immunoblotting was performed as previously described [14]. Briefly, protein samples were separated by SDS-PAGE and then were transferred onto nitrocellulose and probed with primary antibodies. The primary antibodies were detected with a HRP conjugated secondary antibody followed by enhanced chemiluminescence development (Pierce Chemical, Rockford, IL) and light detection on Fuji Super RX Film (Tokyo, Japan). For graphical analysis, sub-saturated band exposures were scanned using a GS-800 calibrated densitometer. Quantification of band intensity was finished by using ImageJ software. For the quantification of the phosphorylation of various proteins, the band intensity of phosphorylated proteins is normalized against the band intensity of non-phosphorylated proteins. Two-tailed Student *t*-tests were completed using MedCalc software. ** indicates $p < 0.01$ and * indicates $p < 0.05$.

2.4. Ras activation assay

Glutathione S-transferase (GST) fused to the Raf Ras binding domain (GST-RBD), precoupled to glutathione–agarose beads in BOS buffer, was added, and the lysates were incubated at 4 °C for 1 h. Beads were collected by centrifugation and washed three times with BOS buffer, and then loading buffer was added. Ras was detected with the monoclonal anti-Ras antibody, followed by a horseradish peroxidase (HRP)-coupled anti-mouse antibody.

Please cite this article as: P. Wee, et al., EGF stimulates the activation of EGF receptors and the selective activation of major signaling pathways during mitosis, Cell. Signal. (2014), http://dx.doi.org/10.1016/j.cellsig.2014.11.030