Detection of O-linked N-acetylgalactosamine post-translational modification of the epidermal growth factor receptor in A431 tumor cells

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ABSTRACT

O-GlcNAcylations are the reversible addition of single O-linked β-N-acetylgalactosamine (O-GlcNAc) moieties to serine or threonine residues of proteins. This post-translational modification has been shown to occur in many nuclear and cytoplasmic proteins [1]. An extensive crosstalk between O-GlcNAcylation and phosphorylation has been described [2]. There are two enzymes implicated in this process: β-N-acetylgalactosaminyl transferase (OGT) adding GlcNAc moieties, and β-N-acetylgalactosaminidase (OGA) removing these moieties [3, 4]. It has been demonstrated using high throughput proteomic analysis that the epidermal growth factor receptor (EGFR) type III of Drosophila melanogaster undergoes O-GlcNAcylation [5]. We present experimental evidences suggesting that the EGFR from human carcinoma epidermal A431 cells is subjected to O-GlcNAcylation. We detected a positive O-GlcNAcylation signal in immunoprecipitated EGFR using immunobot and two distinct specific anti-O-GlcNAc antibodies. Conversely, the presence of O-GlcNAc was detected by immunoblot among the O-GlcNAcylated proteins immunoprecipitated with an anti-O-GlcNAc antibody. These signals were enhanced when Thimet O, a highly specific OGA inhibitor, was present. Most significantly, we detected a positive O-GlcNAcylation signal in immunoprecipitated and -deglucosylated EGFR (PNGase F) from tumoricentric treated cells when metabolically labeled with azido-GlcNAc, biotinylated and labeled with streptavidin-labeled peroxidase. Finally, we performed O-GlcNAcylation assay in vitro using immunoprecipitated EGFR and OGT in the presence of the substrate UDP-GlcNAc, which resulted in the enhancement of the EGFR O-GlcNAcylation signal as detected by immunobot. We conclude that the EGFR from A431 tumor cells is subjected to O-GlcNAcylation and this may regulate the functionality of the receptor.

INTRODUCTION

The EGFR is a transmembrane glycoprotein that belongs to the ErbB receptor tyrosine kinase family. Its structure consists of an extracellular region responsible for ligand binding, a single transmembrane segment and a cytoplasmic region that contains a juxtamembrane domain, a tyrosine kinase domain and a C-terminal tail (Figure 1). Binding of a variety of ligands, including EGF, TGF, or, and HGF among others, to the EGFR leads to the homodimerization of the receptor or heterodimerization with other ErbB family members and activation of its intrinsic tyrosine kinase followed by autophosphorylation at multiple tyrosine residues located in the C-terminal tail. These sites are docking places for different cytoplasmic proteins containing SH2 and PTB adaptor proteins and transcription signaling molecules, activate signaling nodes such as the MAPK, PI3K/AKT, PLCγ, and the STAT pathways (Figure 1). The activation of these signaling pathways results in cell cycle progression/proliferation, cell migration, cell survival and differentiation. EGFR is frequently overexpressed and/or mutated in many solid tumors thus contributing to cancerogenesis [6].

The occurrence of O-GlcNAc at Ser and Thr residues has been recognized as an abundant posttranslational modification of a myriad of cellular proteins, playing important regulatory roles and controlling critical cellular functions such as cell proliferation, apoptosis, cell death, immune response, cell adhesion, cell motility, angiogenesis, and cell survival. O-GlcNAcylations play a role in the development of some neurodegenerative ailments such as Alzheimer’s disease, cancer, diabetes, as well as type 2 diabetes mellitus [7]. In silico analysis suggests that Thr64 and Ser1046/1047 of the human EGFR receptor are potential sites for O-GlcNAcylation, although no experimental evidence has been provided so far to demonstrate this assertion [8] in this report we shall provide proof that indeed the human EGFR could be subjected to O-GlcNAcylation in a tumor cell line.

RESULTS

Figure 3. Detection of O-GlcNAcylated signal in immunoprecipitated EGFR by Western blot. (A) Western blot was performed using anti-EGFR antibody and the immunocomplex was incubated in the absence (-) and presence (+) of PNGase F to remove N-glycans. The samples were immunoblotted (WB) with the anti-O-GlcNAc antibodies CTD110(6) and RL2 (B) as indicated. The PVDF membranes were stripped and probed with an anti-EGFR antibody as loading control. Mock PK (a) was performed using a non-relevant IgG as negative control. Upper and lower arrowheads point the native and N-deglycosylated EGFR, respectively.

Figure 4. Effects of OGT and OGA inhibitors on the EGFR O-GlcNAcylated signal. (A) A431 cells were incubated overnight in the absence (−) and presence (+) of 20 μM Thimet O or 2 mM BADGP as indicated. The samples were immunoprecipitated (IP) using an anti-EGFR antibody and the immunocomplex was incubated in the absence (-) and presence (+) of PNGase F to remove N-glycans, processed by SDS-PAGE and Western blotting (WB) and probed with an anti-O-GlcNAc antibody (RL2). The PVDF membrane was stripped and probed with an anti-EGFR antibody as loading control. (B) The plot presents the mean ± SD of 3) O-GlcNAcylated EGFR from a set of experiments similar to the one shown in A measuring the densitometry of the O-GlcNAcylated band corrected by loading as determined by the Student’s t-test. Upper and lower arrowheads point the native and N-deglycosylated EGFR, respectively. (C) A431 cells were incubated overnight in the absence (−) and presence (+) of 20 μM Thimet O or 2 mM BADGP as indicated. The proteins were immunoprecipitated (IP) using an anti-O-GlcNAc antibody (RL2) and the immunocomplex processed by SDS-PAGE and Western blotting (WB) using an anti-EGFR antibody. A mock IP was performed using a non-relevant IgG as negative control. The heavy chain IgG band stained with Fast Green is shown.

Figure 5. Effects of OGT and OGA inhibitors on cell migration. Artificial wounds were performed in confluent monolayers of A431 cells in the absence (None) or in the presence of the OGT inhibitor BADGP (1 mM) and the OGA inhibitor Thimet O (100 μM). Photographs were taken at different times in order to analyze the dynamics of the wound closure in a Zeiss Cell Observer system. A typical pattern of photographs taken at different times are shown. (B) The plot represents the mean ± SEM (n = 4) of the closing of the wound in the different conditions. Significant differences when comparing the curves using the two-way ANOVA test are shown (**p < 0.001).