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

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ABSTRACT

O-GlcNAcylation is the reversible addition of single *O*-linked β-D-*N*-acetylglucosamine (*O*-GlcNAc) moieties to serine or threonine residues of proteins. This post-translational modification has been shown to occur in many nuclear and cytosolic proteins [1]. An extensive crosstalk between *O*-GlcNAcylation and phosphorylation has been described [2]. There are two enzymes implicated in this process: β-*N*-acetyl-glucosaminyl transferase (OGT) adding GlcNAc moieties, and β-*N*-acetylglucosaminidase (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 epidermoid A431 cells is subjected to *O*-GlcNAcylation. We detected a positive *O*-GlcNAcylation signal in immunoprecipitated EGFR using immunoblot and two distinct specific anti-*O*-GlcNAc antibodies. Conversely, the presence of EGFR was detected by immunoblot among the *O*-GlcNAcylated proteins immunoprecipitated with an anti-*O*-GlcNAc antibody. These signals were enhanced when Thiamet G, a highly specific OGA inhibitor, was present. Most significantly, we detected a positive *O*-GlcNAcylation signal in immunoprecipitated and *N*-deglycosylated EGFR using peptide-*N*-glycosidase F (PNGase F), and from tunicamycin-treated cells when were metabolically labeled with azido-GlcNAc, biotinylated and probed 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 immunoblot. 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 which is responsible for ligand binding, a single transmembrane segment and a cytosolic 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-α, and HB-EGF among others, to the EGFR leads to the homo-dimerization of the receptor or hetero-dimerization with other ErbB family members and activation of its intrinsic tyrosine kinase followed by auto(trans)-phosphorylation at multiple tyrosine residues located in the C-terminal tail. These sites are docking places for different cytosolic proteins containing SH2 and PTB adaptor proteins and transduction complexes to activate signaling routes such as the MAPK, PI_3K/AKT, PLCγ/PKC, 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 [7].

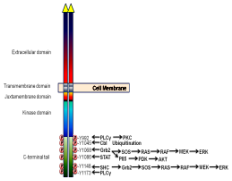

Figure 1. The EGFR signaling pathway

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 (Figure 2). Alterations in the systems involved in protein *O*-GlcNAcylation have been implicated in the development of some neurodegenerative ailments such as Alzheimer's disease, cancer, as well type 2 diabetes mellitus [1].

In silico analysis suggests that Thr654 and Ser1046/1047 of the human EGFR could be targeted by *O*-GlcNAcylation, although no experimental evidence has been provided so far to demonstrate this assertion [6]. In this report we shall provide proof that indeed the human EGFR could be subjected to *O*-GlcNAcylation in a tumor cell line.

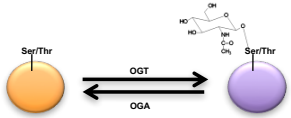

Figure 2. Reversible *O*-GlcNAcylation of Ser/Thr in a target protein.

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RESULTS

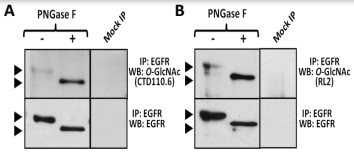

Figure 3. Detection of *O*-GlcNAcylation signal in immunoprecipitated EGFR by Western blot. (*A, B*) The EGFR was immunoprecipitated (IP) from an A431 cell extract using an anti-EGFR antibody and the immunocomplex was incubated in the absence (-) and presence (+) of PNGase F to remove *N*-glycans. The samples were immunoblotted (WB) with the anti-*O*-GlcNAc antibodies CTD110.6 (*A*) and RL2 (*B*) as indicated. The PVDF membranes were stripped and probed with an anti-EGFR antibody as loading control. Mock IP was performed using a non-relevant IgG as negative control. Upper and lower arrowheads point the native and *N*-deglycosylated EGFR, respectively.

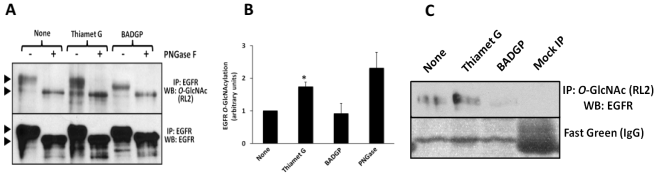

Figure 4. Effects of OGT and OGA inhibitors on the EGFR *O*-GlcNAcylation signal. (*A*) A431 cells were incubated overnight in the absence (*None*) and presence of 20 μM Thiamet G or 2 mM BADGP as indicated. The samples were immunoprecipitated (IP) using an anti-EGFR antibody and the immunocomplex was incubated in the absence (-) and presence (+) of PNGase F to remove *N*-glycans, processed by SDS-PAGE and Western blots (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 ± SEM (n = 3) EGFR *O*-GlcNAcylation 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 total EGFR signal. (*) $p < 0.05$ 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 (*None*) and presence of 20 μM Thiamet G 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 blots (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.

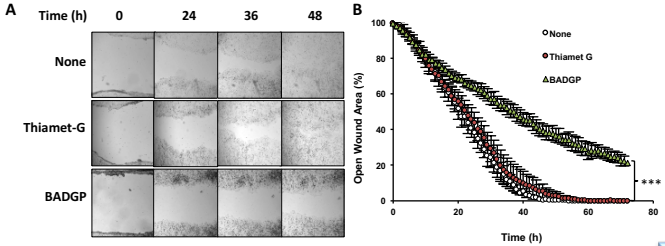

Figure 5. Effects of OGT and OGA inhibitors on cell migration. Artificial wounds were performed in confluent monolayers of A431 cells in the absence (*None*) or in the presence of the OGT inhibitor BADGP (1 mM) and the OGA inhibitor Thiamet G (100 μM). Photographs were taken at different times in order to follow the closure of the wounds using a Zeiss Cell Observer system. (*A*) A typical set 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 (*** $p < 0.0001$).

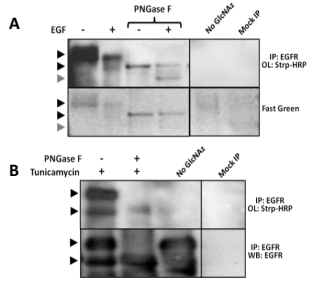

Figure 6. Detection of *O*-GlcNAcylation signal in immunoprecipitated EGFR from azido-GlcNAc-treated cells. (*A*) Serum-starved A431 cells were treated overnight with 40 μM azido-GlcNAc (GlcNAz) and incubated as indicated in the absence (-) and presence (+) of 10 nM EGF during 30 min. Thereafter, the EGFR was immunoprecipitated (IP) and where indicated treated with PNGase F to remove *N*-glycans. The samples were subjected to biotinylation and overlaid with streptavidin-HRP to detect GlcNAz-labeled proteins. Immunoprecipitated EGFR from cells non-treated with GlcNAz (*No GlcNAz*) and a mock IP are shown as negative controls. Protein staining of the immunoprecipitated EGFR band with Fast Green is shown as loading control. (*B*) A431 cells were treated overnight with 1 μg/ml tunicamycin, the EGFR was immunoprecipitated (IP) and where indicated treated with PNGase F to remove potential residual *N*-glycans. The samples were subjected to biotinylation and overlaid with streptavidin-HRP to detect GlcNAz-labeled proteins. Duplicate samples were probed with an anti-EGFR antibody. Immunoprecipitated EGFR from cells non-treated with GlcNAz (*No GlcNAz*) and a mock IP are shown as negative controls. Upper and lower black arrowheads (*A, B*) point the native and *N*-deglycosylated EGFR, respectively, and the gray arrowhead (*A*) points to a partially degraded *N*-deglycosylated EGFR.

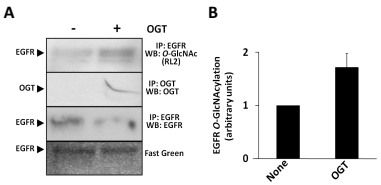

Figure 7. Enhanced *O*-GlcNAcylation of EGFR upon *in vitro* reaction catalyzed by immunoprecipitated OGT. (*A*) OGT and EGFR were independently immunoprecipitated from A431 cells. Thereafter, the immunoprecipitated EGFR was incubated in the absence (-) and presence (+) of immunoprecipitated OGT and the *O*-GlcNAcylation reaction was performed upon addition of UDP-GlcNAc. The samples were immunoblotted (WB) with an anti-*O*-GlcNAc antibody (CTD110.6) as indicated. The EGFR as detected with an anti-EGFR antibody and the heavy chain of IgG stained with Fast Green are shown as loading controls. (*B*) The plot presents the mean ± range EGFR *O*-GlcNAcylation in the absence (*None*) and presence of OGT from two independent experiments similar to the one shown in *A* measuring the densitometry of the *O*-GlcNAcylated EGFR band corrected by loading as determined by protein staining with Fast Green.

MAJOR FINDINGS & CONCLUSION

1. **Removal of *N*-glycans in the EGFR enhances the reactivity of the anti-*O*-GlcNAc antibodies.**
2. **Inhibition of OGA leads to a significant increase in the EGFR *O*-GlcNAcylation signal.**
3. ***N*-deglycosylation did not diminish the azido-GlcNAc signal of the EGFR.**
4. **OGT *O*-GlcNAcylates the EGFR in an *in vitro* assay system.**
5. **We conclude that the human EGFR in A431 tumor cells is subjected to *O*-GlcNAcylation.**