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Direct interaction between surface β1,4-galactosyltransferase 1 and epidermal growth factor receptor (EGFR) inhibits EGFR activation in hepatocellular carcinoma

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ABSTRACT

Our previous studies showed that cell surface β1,4-galactosyltransferase 1 (β1,4GT1) negatively regulated cell survival through inhibition and modulation of the epidermal growth factor receptor (EGFR) signaling pathway in human hepatocellular carcinoma (HCC) SMMC-7721 cells. However, the underlying mechanism remains unclear. Here we demonstrated that β1,4-galactosyltransferase 1 (β1,4GT1) interacted with EGFR *in vitro* by GST pull-down analysis. Furthermore, we demonstrated that β1,4GT1 bound to EGFR *in vivo* by co-immunoprecipitation and determined the co-localization of β1,4GT1 and EGFR on the cell surface via confocal laser scanning microscopy analysis. Finally, using ^{125}I-EGF binding experiments and Western blot analysis, we found that overexpression of β1,4GT1 inhibited ^{125}I-EGF binding to EGFR, and consequently reduced the levels of EGFR dimerization and phosphorylation. In contrast, RNAi-mediated knockdown of β1,4GT1 increased the levels of EGFR dimerization and phosphorylation. These data suggest that cell surface β1,4GT1 interacts with EGFR and inhibits EGFR activation.

1. Introduction

β1,4-Galactosyltransferase 1 (β1,4GT1) is one of the most exhaustively studied mammalian glycosyltransferases responsible for the biosynthesis of disaccharides, oligosaccharides and polysaccharides [1]. The β1,4GT1 gene is unusual because it encodes two protein isoforms generated by differential translation initiation [2,3]. Both isoforms are primarily localized in the trans-Golgi network involved in the biosynthesis of complex-type oligosaccharides. However, a portion of the long, but not the short, isoform of β1,4GT1 is preferentially targeted to the cell surface, where it serves as an adhesion molecule and participates in a number of cellular interactions [4]. Surface-localized glycosyltransferase forms a stable adhesive bond with its glycoside substrate in the extracellular matrix or on an adjacent cell surface. It participates in cellular interactions, including neurite extension, cell growth, sperm-egg interaction, cell spreading, and migration [5].

Our previous studies have shown that altering the expression of cell surface β1,4GT1 modulates cell growth and apoptosis [6–11]. The β1,4GT1 mRNA level was greatly increased during the apoptosis of human HCC SMMC-7721 cells induced by cycloheximide (CHX) [6]. Meanwhile, overexpression of β1,4GT1 enhanced the susceptibility of human HCC SMMC-7721 cells to CHX triggered apoptosis [7]. Further studies showed that β1,4GT1 promoted apoptosis induced by CHX in human HCC SMMC-7721 cells through modulation of the EGFR pathway, including the PI3K-PKB/Akt and Ras-MAPK cascades [8]. However, the mechanisms underlying the role of β1,4GT1 in EGFR mediated cell survival and apoptosis remain unclear.

EGFR is overexpressed in various kinds of cancer cells and participates in the regulation of cancer invasion, metastasis, and angiogenesis [12]. It transduces cell signaling through ligand (EGF)-induced receptor dimerization, which then initiates its tyrosine kinase activity to direct the malignant behavior of cancer [13]. Overexpression of EGF and EGFR has been reported in human HCC, and is associated with the late-stage of disease, increased cell proliferation and degree of tumor differentiation [14,15]. We previously demonstrated that cell surface β1,4GT1 might play a role in EGFR mediated cell survival and apoptosis in human HCC SMMC-7721 cells [8]. Here we report that cell surface β1,4GT1 interacts directly with EGFR and inhibits EGFR activation in human HCC cells.

2. Materials and methods

2.1. Cells and reagents

The human HCC SMMC-7721 cell line was obtained from the Institute of Cell Biology, Academic Sinica. RPMI-1640 medium

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was purchased from Gibco/BRL (Eggenstein, Germany); monoclonal mouse anti-β1,4GT1 (long isoform) antibody, from R&D Systems (Minneapolis, USA); monoclonal rabbit anti-EGFR antibody, from Millipore (Bedford, MA, USA); mouse anti-glutathione S-transferase (GST) and anti-GAPDH antibodies, from Kangwei Biotechnology (Beijing, China); ECL Western Blotting Substrate System, from Pierce (Rockford, IL, USA). The human β1,4GT1 cDNA was cloned into pcDNA3.1 (Invitrogen) vectors as previously described [6]. Construction of recombinant pSilencer expressing β1,4GT1 siRNA was generated as reported [7].

2.2. Tissue samples

The pathological samples were retrieved from the Department of Pathology, Zhongshan Hospital (Shanghai, China). All experiments using human subjects were performed in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Fudan University.

2.3. Cell culture and stable transfectants

The human HCC SMMC-7721 cells were cultured in RPMI medium 1640 (Thermo, USA), supplemented with 15% fetal bovine serum (FBS) (GIBCO, Austria) at 37 °C in a humidified atmosphere containing 5% CO_2. Stable transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Monoclonal cells were then selected, cloned, and screened by adding G418.

2.4. Purification of GST fusion proteins

The sequence coding for β 1,4GT1 was amplified by PCR from the purified pcDNA3.1-β1,4GT1 plasmid and gene-specific primers with *BamH*I and *EcoR*I restriction enzyme sites added for unidirectional cloning. The primer sequences are listed as follows:

Forward: 5′-ATCGGATCCATGAGGTTTCGTGAGCAG-3′

Reverse: 5′-ACGGAATTCCTATCTCGGTGTCCCGATG-3′

The recombinant plasmid pGEX-4T-2-β1,4GT1 was checked for accurate insertion via restriction enzyme analysis. The GST-tagged protein was expressed in *Escherichia coli* (DH5α), which was grown in Luria–Bertani (LB) medium supplemented with ampicillin (100 μg/ml) at 37 °C and induced at OD_{600} 0.6 with 0.2 mM isopropyl β-D-thiogalactoside (IPTG). The bacterial pellet was suspended in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl) and sonicated. The supernatant was collected by centrifugation at 15,000 rpm for 45 min. GST-tagged β 1,4GT1 was applied to a glutathione Sepharose 4B column (GE Healthcare, USA), which was treated with wash buffer (20 mM Tris–HCl, pH 7.5, 500 mM NaCl). The adsorbed protein was eluted with buffer (20 mM glutathione, 50 mM Tris–HCl, pH 8.5). The eluate was loaded onto a pre-equilibrated ion exchange Q-Sepharose column (Amersham Biosciences, NJ, USA) and purified using the Äkta purifier 100 system (GE Healthcare, Uppsala, Sweden).

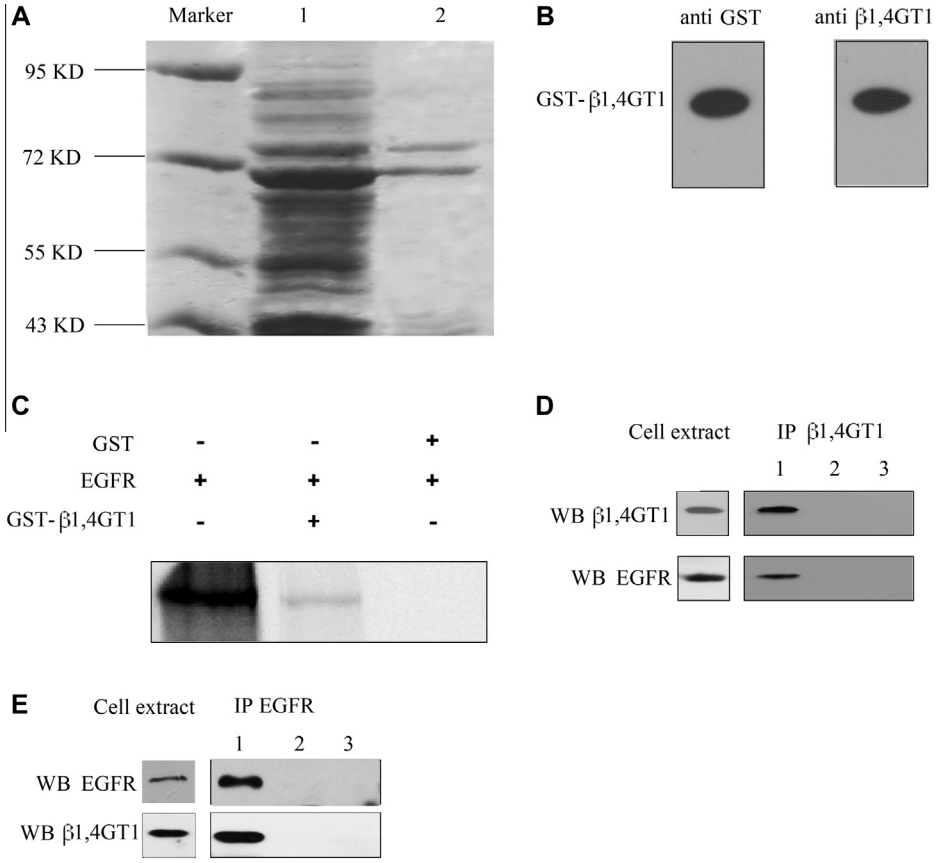

Fig. 1. Interaction of β1,4GT1 and EGFR *in vitro* and *in vivo*. (A) GST-β1,4GT1 protein was expressed in *E. coli* (DH5α). Lane 1, bacterial total soluble protein; lane 2, recombinant GST-β1,4GT1 purified by GST Sepharose 4B affinity chromatography and Äkta purifier. (B) Immunoblotting of purified recombinant GST-β1,4GT1 fusion protein using anti-GST and anti-β1,4GT1 antibodies. (C) Interaction between β1,4GT1 and EGFR *in vitro*. EGFR alone (input for the pull-down assay) is shown in lane 1. *In-vitro*-translated EGFR was incubated with GST (lane 3) or GST-β1,4GT1 protein (lane 2). The protein mixtures were bound to glutathione Sepharose beads and washed before analysis by autoradiography. (D and E) Interaction between β1,4GT1 and EGFR *in vivo*. Human hepatocellular carcinoma tissue lysates were co-immunoprecipitated with: lane 1, anti-β1,4GT1/anti-EGFR antibody; lane 2, isotype control antibody; lane 3, PBS and immunoblotted with the indicated antibody.

2.5. In vitro binding assays

Full-length EGFR was transcribed and translated *in vitro* using [^{35}S] methionine and the TNT-coupled reticulocyte lysate system (Promega, Madison, WI, USA). In briefly, 1 μl plasmid DNA (1 μg/μl) was added to the TNT-coupled reticulocyte lysate system together with 2 μl [^{35}S] methionine, 12.5 μl TNT lysate, 0.5 μl RNA polymerase, 0.5 μl ribonuclease inhibitor, 2.5 μl canine microsomal membranes, 0.5 μl amino acid mixture (minus methionine), 0.5 μl reaction buffer and 5 μl nuclease-free water. The mixtures were incubated at 30 °C for 90 min. A 10 μl aliquot of the translation mix was added to a comparable amount of immobilized GST or GST fusion proteins. The binding was allowed to occur at room temperature for 2 h in 0.3 ml of binding buffer (50 mM HEPES, pH 7.5; 200 mM NaCl, 0.5% NP-40, 1% BSA, 1 mM DTT, 5 mM EDTA, 0.005% SDS, and aprotinin 2 mg/ml). Beads were then washed four times with 1 ml of binding buffer. The bound proteins were eluted by boiling in SDS sample buffer, separated on SDS–PAGE, and visualized by autoradiography. The *in vitro*-translation product (10 μl) was also loaded onto the gel to confirm the size of the input protein.

2.6. Co-immunoprecipitation (Co-IP) and Western blotting

Co-IP and Western blotting were carried out as described previously [16]. In brief, the cells and tissues were lysed in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 with protease inhibitor). Protein concentrations were determined using the BCA protein assay (Biotech, Shanghai, China). Equal amounts of proteins were subjected to SDS–PAGE, electroblotted onto PVDF membranes, incubated with specific antibodies and detected with ECL. For co-IP, the lysates were precleared with protein A-Sepharose beads at 4 °C for 8 h, and then incubated with protein A-agarose beads (Roche) and antibodies at 4 °C overnight. The precipitates were pelleted and washed three times with the lysis buffer before elution in SDS-loading dye and Western blotting. For analysis of EGFR dimerization on the cell surface, cells were starved in serum-free medium overnight, followed by stimulation with EGF (10 ng/ml) for 5 min. Cross-linking reactions were performed by the addition of 1 mM BS3 followed by incubation at RT for 20 min, and termination by the addition of 50 mM Tris–HCl (pH 7.4). The samples were analyzed by SDS–PAGE and Western blotting.

2.7. Immunofluorescence

Human HCC SMMC-7721 cells were fixed in ice-cold methanol for 1 h and blocked in PBS containing 10% normal blocking serum followed by overnight incubation at 4 °C with the primary antibodies: monoclonal mouse anti-β1,4GT1 (long isoform) antibody and monoclonal rabbit anti-EGFR antibody. After overnight incubation, the coverslips were rinsed three times in PBS and incubated at 37 °C for 1 h with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG and rhodamine-conjugated anti-rabbit IgG. The slides were then stained with DAPI, washed with PBS-T, mounted, and analyzed by confocal laser scanning microscopy (Leica TCS, Germany).

2.7.1. ^{125}I-labeled EGF binding experiments

^{125}I-EGF binding experiments were performed as previously described [17,18]. EGF (Biomedical Technologies) was labeled with ^{125}I using the Iodogen method (Pierce) according to the manufacturer's instructions. Human HCC SMMC-7721 cells that were stably transfected with pcDNA3.1-β1,4GT1 or pcDNA3.1 empty vector were used to determine the time-course (5–240 min) of ^{125}I-EGF binding. Cells were incubated at 4 °C in 1 ml of Hepes binding buffer containing ^{125}I-EGF (2 ng/ml) in the presence or absence of 125-fold excess of nonradioactive EGF. To determine the displacement curves of ^{125}I-EGF binding, cells were incubated with ^{125}I-EGF (5 ng/ml) for 1 h at room temperature in the presence of increasing concentrations of unlabeled EGF. The treated cells were lysed. The radioactive contents of the samples were determined using a scintillation counter. The time-course of EGF binding and the displace-

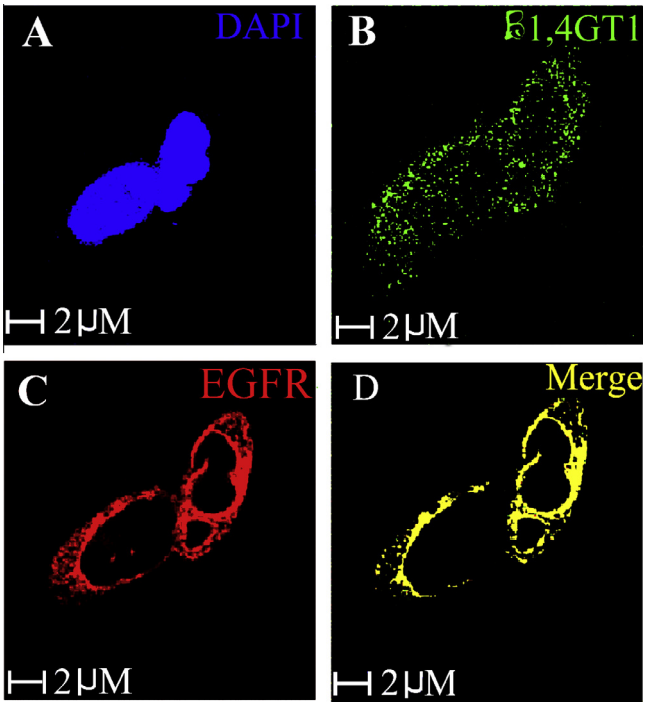

Fig. 2. β1,4GT1 co-localizes with EGFR. Hepatocellular carcinoma SMMC-7721 cells were fixed and stained with anti-β1,4GT1 and anti-EGFR antibodies. (A) Nuclei were visualized by DAPI. For double immunofluorescence staining, the slides were incubated with (B) FITC-conjugated anti-mouse IgG and (C) Rhodamine-conjugated anti-rabbit IgG before visualization by confocal laser scanning microscopy. (D) Merged images showing protein co-localization.

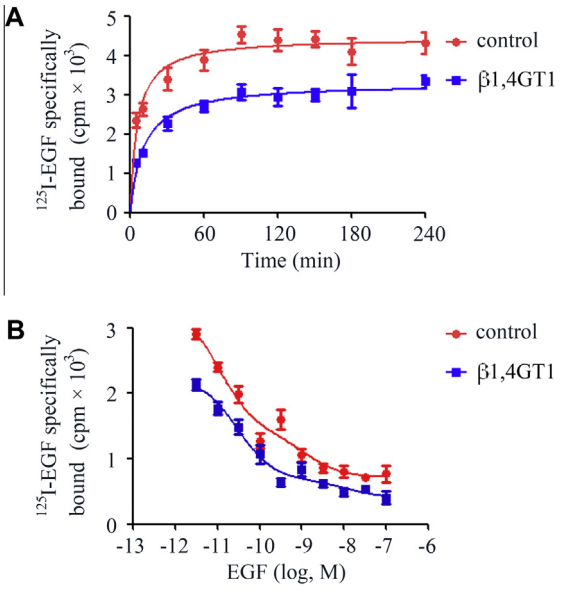

Fig. 3. The effect of β1,4GT1 on ^{125}I-EGF binding. (A) Time-course of ^{125}I-EGF binding to control and β1,4GT1 transfected hepatocellular carcinoma SMMC-7721 cells. (B) Competition binding of ^{125}I-EGF to control and β1,4GT1 transfected hepatocellular carcinoma SMMC-7721 cells in the presence of increasing concentrations of native EGF. Means ± SEM from four independent experiments are shown.

ment curves of ^{125}I-EGF binding were calculated using Prism version 4.0 software (GraphPad).

2.8. Statistical analysis

All data are expressed as mean ± SEM. Differences between the groups were analyzed by ANOVA followed by a Newman–Keuls test using GraphPad Prism version 4.0. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. β1,4GT1 binds specifically to EGFR both in vitro and in vivo

β1,4GT1 was cloned into the pGEX-4T-2 expression vector, and expressed in *E. coli* (DH5α) after IPTG induction. The protein was purified by GST Sepharose 4B affinity chromatography and the Äkta purifier 100 system (Fig. 1A). The purified GST-β1,4GT1 fusion protein was further validated by immunoblotting with anti-GST or anti-β1,4GT1 antibodies (Fig. 1B). EGFR was transcribed and trans-

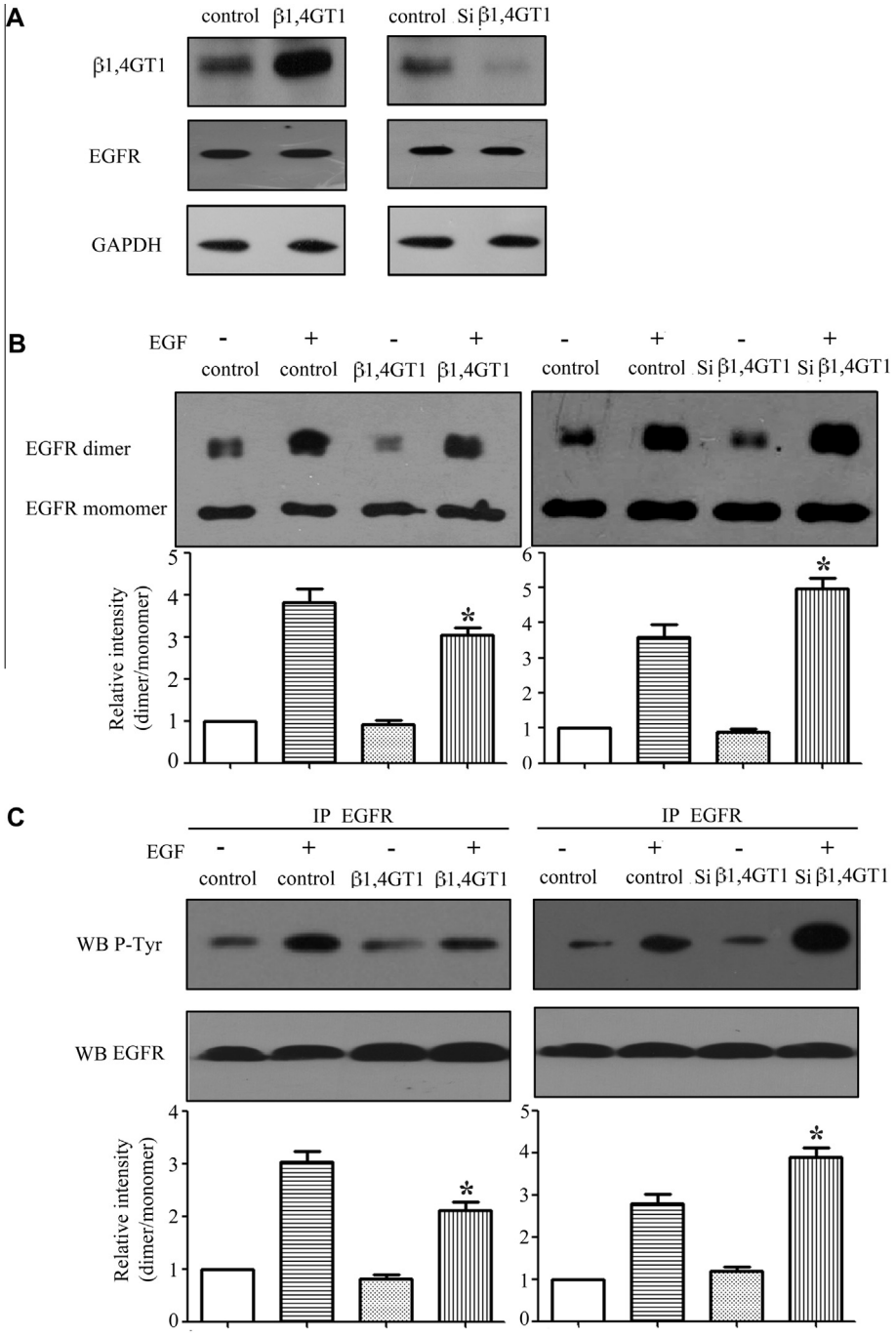

Fig. 4. The effect of β1,4GT1 on EGF-induced EGFR dimerization and phosphorylation. (A) The effect of β1,4GT1 overexpression and RNAi on EGFR expression in hepatocellular carcinoma SMMC-7721 cells. (B) EGF (10 ng/ml) induced dimerization of EGFR. Quantitative results calculated by EGFR dimer:monomer ratio in each sample normalized with against the ratio of 7721-control without EGF treatment. The 7721-control cells were transfected with pcDNA3.1 vector or pSilencer 2.1-U6 neo vector. Means ± SEM from three independent experiments are shown. (C) EGF (10 ng/ml) induced EGFR phosphorylation. The relative intensity indicated below was calculated as the phospho-EGFR:EGFR ratio and normalized against the ratio of 7721-control without EGF treatment. Means ± SEM from three independent experiments are shown. $^{*}P < 0.05$ compared with 7721-control treated with EGF.

lated *in vitro* using [^{35}S] methionine and the TNT-coupled reticulocyte lysate system. Pull-down analysis was performed using recombinant GST-β1,4GT1 protein and *in vitro*-translated radiolabelled EGFR protein. GST-β1,4GT1 (Fig. 1C, lane 2), but not GST (Fig. 1C, lane 3), bound to EGFR, which suggested a direct interaction between β1,4GT1 and EGFR *in vitro*.

Previous studies indicated that β 1,4GT1 negatively regulates cell survival possibly through inhibition and modulation of the EGFR signaling pathway in human HCC cell lines [8]. Our investigations demonstrated co-immunoprecipitation of β1,4GT1 and EGFR in human HCC tissues (Fig. 1D), and vice versa (Fig. 1E). To verify the stringency of co-IP analysis, parallel experiments were conducted in which the co-immunoprecipitating antibody was replaced with an isotype-matched control antibody or PBS. Moreover, we also observed interaction between β1,4GT1 and EGFR in human HCC SMMC-7721 cells (data not shown).

3.2. β1,4GT1 co-localizes with EGFR

Immunofluorescence analysis was conducted to determine whether β1,4GT1 and EGFR are co-localized in the same cellular compartment in human HCC SMMC-7721 cells using monoclonal mouse anti-β1,4GT1 and monoclonal rabbit anti-EGFR antibodies, respectively. The localization of the two proteins were subsequently visualized using FITC and rhodamine-conjugated secondary antibodies under confocal laser scanning microscopy (Fig. 2A–C). β1,4GT was found to be distributed at the cell surface, and also in the intracellular organelles. EGFR was found to be mainly localized in the plasma membrane. Co-localization of the two proteins on the cell surface was shown by overlapping fluorescent signals (Fig. 2D).

3.3. β1,4GT1 inhibits ^{125}I-EGF binding

To evaluate the effect of β1,4GT1 on the ligand binding characteristics of cell surface EGFRs, human HCC SMMC-7721 cells were stably transfected with either pcDNA3.1-β1,4GT1 or pcDNA3.1 empty vector. The quantitative binding experiments were then conducted. The time-course of ^{125}I-EGF binding to human HCC SMMC-7721 cells is shown in Fig. 3A. Overexpression of β1,4GT1 significantly inhibited ^{125}I-EGF binding at different time-points. The displacement curves of ^{125}I-EGF binding to control and β1,4GT1 overexpressing cells is shown in Fig. 3B. The binding data were further analyzed using GraphPad Prism software to derive the *Kd* for EGF binding. In control (empty vector-transfected) cells, Kd_1 (for the high affinity site) and Kd_2 (for the low affinity site) values were 0.39 nM and 2.25 nM, respectively. In β1,4GT1 transfected cells, Kd_1 was 0.71 nM and Kd_2 was 3.34 nM. These experiments revealed the inhibitory effect of β1,4GT1 on the binding affinity of EGF to EGFR.

3.4. β1,4GT1 inhibits EGFR activation

To determine the effect of β1,4GT1 on EGFR activation, we analyzed EGFR dimerization and phosphorylation upon EGF stimulation. We first analyzed the EGFR protein levels in human HCC SMMC-7721 cells. Overexpression or RNA interference of β1,4GT1 did not affect total EGFR levels (Fig. 4A). We next investigated EGFR dimerization upon EGF stimulation in human HCC SMMC-7721 cells. As shown in Fig. 4B, overexpression or RNA interference of β1,4GT1 significantly inhibited or promoted, respectively, the EGF-induced EGFR dimerization. Further analysis of phosphorylation status showed weaker EGFR tyrosine phosphorylation in β1,4GT1 overexpressing cells and stronger EGFR tyrosine phosphorylation in β1,4GT1-siRNA cells compared with control (Fig. 4C), which was consistent with the dimerization results.

4. Discussion

The diverse biological functions of surface β1,4GT1 indicate that it is capable of interacting with multiple extracellular ligands or other intracellular proteins. On the cell surface, the catalytic region of β1,4GT1 is exposed. Therefore surface β1,4GT1 can recognize and bind to terminal GlcNAc residues, which are found in a variety of extracellular and cell surface glycoproteins and glycolipids, such as laminin1, zona glycoprotein ZP3 and E-cadherin [19]. EGFR has been also reported to be expressed as a 170-kDa glycoprotein with GlcNAc terminus [20]. In this study, GST pull-down assays showed that cell surface β1,4GT1 interacted with glycoprotein EGFR *in vitro*. We also demonstrated that cell surface β 1,4GT1 was associated with EGFR *in vivo* by co-immunoprecipitation analyses in human HCC tissues. Moreover, immunofluorescence analysis showed that β1,4GT1 co-localized with EGFR in human HCC SMMC-7721 cells, which is consistent with previous studies reporting that co-localization of β1,4GT and EGFR in a lipid raft membrane compartment [21,22]. Taken together, these data indicate that cell surface β1,4GT1 interacts with EGFR in human HCC.

EGFR consists of an extracellular receptor domain that binds EGF, a transmembrane region, and an intracellular domain with tyrosine kinase function. The 621 amino acid residues of the extracellular domain can be subdivided into four subdomains: two large (L1 and L2) and two small (S1 and S2). Folding of the L1, S1, and L2 domains forms a potential EGF binding pocket [12]. It has been reported that the interactions between EGFR and other cell surface receptors such as the estrogen receptor (ER) affect the conformation of EGFR, and consequently change the affinity of EGFR for EGF [23,24]. The cross-talk between EGFR and other cell surface receptors could lead to EGFR downstream signaling changes [25,26]. A growing body of evidence indicates that cell surface β1,4GT1 functions as a receptor that binds certain substrates and mediates cell–cell and cell–matrix interactions [5]. Recent data obtained in our laboratory indicated a significant role of β1,4GT1 in the promotion of apoptosis in human HCC SMMC-7721 cells [6–8]. Interestingly, in this study we found β1,4GT1 inhibited ^{125}I-EGF binding to EGFR in the same cell line. It is well established that EGF binding promotes EGFR dimerization and consequently activates intracellular protein tyrosine kinase [27]. Further studies showed that β1,4GT1 inhibited EGFR dimerization and tyrosine phosphorylation in human HCC SMMC-7721 cells. All these data suggest an inhibitory role for β1,4GT1 in EGFR signaling in human HCC.

β1,4GT1 acts as a cell surface receptor that negatively regulates the survival of mammary epithelial cells and embryo fibroblast cells. Overexpression of surface β1,4GT1 directly affects cell proliferation and apoptosis. Previously, we reported that two PI3K inhibitors LY294002 and wortmannin upregulated β1,4GT1 expression and consequently sensitized human HCC SMMC-7721 cells to CHX-induced apoptosis [10]. Here, we further demonstrated that β1,4GT1 interacted with EGFR and inhibited EGFR activation in human HCC. In combination, these findings suggest that β1,4GT1 may serve as a potential therapeutic target for the treatment of HCC.

Acknowledgments

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