Knockdown of GnT-Va expression inhibits ligand-induced downregulation of the epidermal growth factor receptor and intracellular signaling by inhibiting receptor endocytosis

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Changes in the expression of N-glycan branching glycosyltransferases can alter cell surface receptor functions, involving their levels of cell surface retention, rates of internalization into the endosomal compartment, and subsequent intracellular signaling. To study in detail the regulation of signaling of the EGF receptor (EGFR) by GlcNAcb(1,6)Man branching, we utilized specific siRNA to selectively knockdown GnT-Va expression in the highly invasive human breast carcinoma line MDA-MB231, which resulted in the attenuation of its invasiveness-related phenotypes. Compared to control cells, ligand-induced downregulation of EGFR was significantly inhibited in GnT-Va-suppressed cells. This effect could be reversed by re-expression of GnT-Va, indicating that changes in ligand-induced receptor downregulation were dependent on GnT-Va activity. Knockdown of GnT-Va had no significant effect on c-Cbl mediated receptor ubiquitination and degradation, but did cause the inhibition of receptor internalization, showing that altered signaling and delayed ligand-induced downregulation of EGFR expression resulted from decreased EGFR endocytosis. Similar results were obtained with HT1080 fibrosarcoma cells treated with GnT-Va siRNA. Inhibited receptor internalization caused by the expression of GnT-Va siRNA appeared to be independent of galectin binding since decreased EGFR internalization in the knockdown cells was not affected by the treatment of the cells with lactose, a galectin inhibitor. Our results show that decreased GnT-Va activity due to siRNA expression in human carcinoma cells inhibits ligand-induced EGFR internalization, consequently resulting in delayed downstream signal transduction and inhibition of the EGFR-induced, invasiveness-related phenotypes.

Keywords: EGFR/endocytosis/GnT-V/N-glycan

Introduction

There is accumulating evidence that aberrant N-glycosylation of cell surface receptors, including both cell adhesion molecules and growth factor receptors, promotes tumor progression. Several recent reports have shown that changes in N-glycan structures on specific receptors was associated with abnormal receptor-mediated, invasive phenotypes by affecting cell adhesion, migration, cell survival, and tumorigenesis (Yoshimura et al. 1996; Guo et al. 2002, 2003; Isaji et al. 2004; Partridge et al. 2004; Seales et al. 2005). N-Acetylglucosaminyltransferase Va (GnT-Va or Mga5a, EC 2.4.1.155), a rate-limiting and oncogene-regulated enzyme in the processing of multiantennary N-glycans during glycoprotein biosynthesis, catalyzes the formation of [GlcNAcb(1,6)Man] branches on N-glycans (Brockhausen et al. 1988; Hakomori 2002). Both in vitro and in vivo studies have implicated GnT-Va in regulating tumor invasiveness and, in some cases, metastatic potential (Demetriou et al. 1995; Seelentag et al. 1998; Granovsky et al. 2000; Yamamoto et al. 2000; Guo et al. 2002, 2003; Partridge et al. 2004; Handerson et al. 2005). Multiple cell surface receptors have been identified as substrates of GnT-Va, including integrins (Demetriou et al. 1995; Guo et al. 2002), cadherins (Guo et al. 2003; Vagin et al. 2008), and growth factor receptors (Guo et al. 2004, 2007; Partridge et al. 2004), and the glycosylation of these receptors by GnT-Va has been shown to be linked to invasive phenotypes.

The human epidermal growth factor receptor (EGFR) contains 12 putative N-glycosylation sites located in extracellular domain I–IV (Ullrich et al. 1984), and N-linked glycosylation of EGFR appears to be essential for its functions, especially the glycosylation in domain III, the major binding site for EGF and TGFα (Greenfield et al. 1989; Lemmon et al. 1997; Tsuda et al. 2000). Studies have shown that EGFR function can be modulated by changes in GnT-Va-related N-glycan expression. The overexpression of GnT-Va in human hepatocarcinoma cells, for example, caused aberrant N-glycosylation of EGFR and increased MAPK signaling mediated by EGF (Guo et al. 2004). We expressed small interfering RNA (siRNA) directed toward GnT-Va transcripts in MDA-MB231 human breast carcinoma cells and found that knockdown of GnT-Va by siRNA expression caused reduced N-linked β(1,6)-branching on EGFR and a significant inhibition of EGF-stimulated cell detachment from matrix, but without affecting the receptor’s ability to bind the ligand (Guo et al. 2007). Moreover, knockdown of GnT-Va also decreased EGF-mediated activation of the tyrosine phosphatase SHP-2, which consequently inhibited the EGFR-mediated dephosphorylation of focal adhesion kinase (FAK), consistent with the attenuation of invasiveness-related phenotypes that included decreased actin rearrangement and cell motility (Guo et al. 2004); Seales et al. 2005).
Interestingly, in polyoma middle T-induced mouse mammary tumor cells from a GnT-Va null background, defective N-glycosylation of EGFR was reported to result in a higher level of EGFR colocalization with EEA-1, an early endosomal marker, suggesting that altered N-glycans on EGFR may result in increased receptor endocytosis when no exogenous EGF is used to induce EGF signaling (Partridge et al. 2004). In these cells, a reduction in EGRF binding to the galletin lattice allowed an increased association with stable caveolin-1 cell surface microdomains that suppresses EGF signaling (Lajoie et al. 2007).

Epidermal growth factor receptor is a member of ErbB family (ErbB1–4) of receptor tyrosine kinases (RTKs) that mediates cellular responses to EGF and transforming growth factor α (TGFα) and plays a crucial role in promoting tumor cell motility and invasion (Sebastian et al. 2006). Upon EGF binding, EGFR dimerizes and becomes autophosphorylated at multiple tyrosine sites within its cytoplasmic tail, C-Cbl, a ubiquitin ligase that mediates ligand-induced ubiquitination of EGFR, is recruited to phosphorylated tyrosines 1045 and 1086 in the carboxy terminal region of the EGFR in response to EGF stimulation (Joazeiro et al. 1999; Levkowitz et al. 1999; Waterman et al. 2002). Receptor ubiquitination via c-Cbl is believed to play a crucial role in regulating endocytic trafficking and degradation of EGFR (Haglund et al. 2003; Mosesson et al. 2003). The ubiquitinated EGFR is then rapidly internalized and transported via clathrin- and/or caveolin-mediated endocytic processes to early endosomes (Sigismund et al. 2005), where the EGFR can be further sorted to the lysosomes, resulting in receptor degradation and termination of receptor signaling. This process is known as ligand-induced EGFR downregulation and plays a key role in regulating receptor signaling intensity after EGF stimulation. Alternatively, internalized EGFR in endosomes can be recycled back to the plasma membrane for continued binding and signaling (Ravid et al. 2004).

The present study investigates how decreased levels of GnT-Va-modified N-glycan expression affect EGF-mediated, invasiveness-related phenotypes using two human tumor cell lines. Unexpectedly, we found that the decreased expression of GnT-Va, resulting from expressing siRNA directed to GnT-Va transcripts, inhibited ligand-induced receptor downregulation of EGFR. Knockdown of GnT-Va had no effect on Cbl-mediated receptor ubiquitination and degradation, however, but EGF-stimulated EGFR internalization was significantly inhibited in GnT-Va-suppressed cells. Our results suggested that reduced GnT-Va expression inhibited EGF-mediated extracellular signaling by affecting EGFR internalization, resulting in reduced invasiveness-related behaviors mediated by EGF. The involvement of galectins in regulating EGFR endocytosis in the GnT-Va knockdown cells was also investigated.

Results

GnT-Va knockdown inhibits ligand-induced downregulation of the epidermal growth factor receptor

To study in more detail the mechanisms of how altered N-glycosylation of EGFR has inhibitory effects on receptor-mediated signaling transduction, the effect of GnT-Va siRNA expression on EGF-induced EGFR endocytosis was investigated using MDA-MB231 cells. Consistent with previous experiments (Guo et al. 2007), GnT-V activity in GnT-Va knockdown cells (62.5 ± 9.1 pmol/h/mg) was significantly reduced compared with control cells (143 ± 10.5 pmol/h/mg) and GnT-Va knockdown cells showed a significant decrease in L-PHA binding (Figure 1A), demonstrating an inhibition of GnT-Va activity and N-linked β(1,6) branching caused by the expression of GnT-Va siRNA. A similar reduction in β(1,6) branching of EGFR was also observed in GnT-Va knockdown cells, as we reported before (Guo et al. 2007), using L-PHA precipitation, blotting, and anti-EGFR staining (data not shown). Both control and GnT-Va knockdown cells were stimulated with EGF for short and long terms, and ligand-induced receptor downregulation was determined by Western blotting after SDS–PAGE using an anti-EGFR antibody. As shown in Figure 1B, a decrease in EGFR levels was observed as early as 15 min after EGF stimulation in control cells, and this decrease became more significant after 60 min of EGF treatment. However, the EGF-induced downregulation of EGFR was inhibited in GnT-Va knockdown cells. This impaired ligand-induced downregulation of EGFR was also observed in knockdown cells when stimulated with EGF overnight (18 h), detected using total cell lysates (Figure 1C) and by cell surface labeling (Figure 1D). When control cells were pretreated with swainsonine, which inhibits Golgi α-mannosidase II and ultimately causes the inhibition of N-linked β(1,6) oligosaccharide expression upstream of the action of GnT-V, ligand-induced downregulation of EGFR was inhibited (Figure 1E). When GnT-Va cDNA was reintroduced into siRNA-knockdown cells, GnT-Va activity showed a significant increase (Guo et al. 2007) and EGF-induced downregulation of EGFR was promoted (Figure 1E). These results confirm the involvement of N-linked β(1,6) branching in regulating EGF-induced receptor downregulation.

To determine whether the inhibition of ligand-induced receptor downregulation in knockdown cells resulted in increased receptor stability, the turnover of EGFR was determined after EGF stimulation. Serum-starved cells were treated with cycloheximide (20 μg/mL), stimulated with EGF for different times, and the total amounts of EGFR were detected by immunoblotting with an anti-EGFR antibody. As shown in Figure 1F, the turnover of EGFR was detected with a half-life of less than 4 h after EGF treatment, while the turnover of EGFR was markedly inhibited with a half-life of EGFR not measurable within the 12 h treatment of cycloheximide. These results indicated that knockdown of GnT-Va inhibited receptor turnover, consistent with the inhibition of ligand-induced receptor downregulation in these cells.

To test whether the observed lack of EGF-induced EGFR downregulation caused by knockdown of GnT-Va was cell-type specific, we chose human fibrosarcoma HT1080 cells, which also express high levels of EGFR, to confirm the results obtained with MDA-MB231 cells. GnT-V activity was reduced in GnT-Va knockdown HT1080 cells (108 ± 12 pmol/h/mg), compared with control cells (180 ± 20 pmol/h/mg). GnT-Va knockdown HT1080 cells showed decreased L-PHA binding (Figure 2A, left panel), indicating the suppression of the expression of β(1,6) branching due to reduced activity of GnT-V caused by the expression of GnT-Va siRNA in these cells. Like MDA-MB231 cells, GnT-Va knockdown HT1080 cells also showed a significant decrease in β(1,6) branching of EGFR detected by using L-PHA precipitation (Figure 2A, right panel). When treated with EGF for different times, ligand-induced
receptor downregulation of EGFR was observed in control cells, as shown in Figure 2B, while in the GnT-Va knockdown HT1080 cells, EGFR downregulation was delayed and less pronounced. Consistent with delayed receptor downregulation, EGFR from GnT-Va knockdown cells showed increased half-life of receptor (Figure 2C), detected by treatment of cells with cycloheximide. These results were similar to those obtained from MDA-MB231 cells and confirmed the effects of knockdown of GnT-Va on ligand-induced EGFR downregulation in human cancer cells.

Ligand-induced receptor dimerization, autophosphorylation, and ubiquitination are not affected by knockdown of GnT-Va

Upon EGF stimulation, EGFR undergoes rapid dimerization, activation of its intrinsic tyrosine kinase activity, autophosphorylation, and ubiquitination, all of which regulate ligand-induced downregulation of EGFR (Joazeiro et al. 1999; Levkowitz et al. 1999; Waterman et al. 2002; Wang et al. 2005; Padron et al. 2007; Grandal and Madshus 2008). To examine whether knockdown of GnT-Va affected ligand-induced receptor dimerization, cells were starved, stimulated with EGF, and cell surface proteins were then chemically crosslinked using BS3 treatment. After EGF was pulled-down with an anti-EGFR antibody, EGFR dimerization was detected by immunoblotting after SDS–PAGE. As shown in Figure 3A, dimerized EGFR with an approximate molecular weight of \( \sim 340 \) kDa was observed after EGF stimulation of both cell lines. In GnT-Va knockdown cells, the level of dimerized EGFR was not decreased and even showed a slight increase. To detect ligand-induced autophosphorylation of EGFR, cells were stimulated with EGF at different times and levels of phospho-EGFR were detected. Decreased phospho-EGFR was not observed in GnT-Va knockdown cells after 20 and 60 min of EGF treatment compared to control cells, however, suggesting that the slower dephosphorylation of EGFR in GnT-Va knockdown cells resulted from inhibited ligand-induced receptor downregulation. Impaired ligand-induced downregulation of EGFR caused by knockdown of GnT-Va, therefore, was not due to defective dimerization nor to defective autophosphorylation of EGFR.
Fig. 2. GnT-Va knockdown inhibits ligand-induced downregulation of the epidermal growth factor receptor in HT1080 cells. (A) GnT-Va-modified N-glycans were detected using cell lysates by lectin-blotting with L-PHA (left panel). Cells were lysed for lectin precipitation (LP) using L-PHA followed by SDS–PAGE, blotting, and detection using anti-EGFR (right panel). As a control, the levels of EGFR was detected using whole cell lysates; *, non-specific band. (B) Cells were starved and stimulated with EGF (100 ng/mL) for indicated times. Cell lysates were prepared and subjected to SDS–PAGE and Western blotting to detect EGFR and ERK (top and bottom panels). The percentage of EGFR remaining represents the amount of EGFR over the amount of EGFR without treatment (middle panel). (C) Subconfluent cells were treated with cycloheximide (20 μg/mL) and with EGF (50 ng/mL) for the indicated times, and cell lysates were subjected to SDS–PAGE and Western blotting for EGFR (top panel). The percentage of EGFR remaining represents the amount of EGFR remaining after the treatment of cycloheximide over the amount of EGFR without treatment (bottom panel).

The autophosphorylated tyrosine residues within the cytoplasmic tail of the dimerized, activated EGFR mediate the recruitment of signaling adaptors, including Grb2, Shc, and c-Cbl (Pawson 1995, 1997; Yarden and Sliwkowski 2001). c-Cbl is a ubiquitin E3 ligase which initiates the ubiquitination of EGFR after being recruited to EGFR, a process that targets activated EGFR to lysosomes for degradation (Joazeiro et al. 1999; Levkowitz et al. 1999; Waterman et al. 2002). Thus, we next investigated whether or not knockdown of GnT-Va caused defects in ubiquitination of EGFR which may account for the impaired ligand-induced receptor downregulation. Compared with control cells, the total levels of c-Cbl protein were not affected before and after EGF stimulation in GnT-Va knockdown cells (Figure 3C). To analyze Cbl/EGFR interactions, after EGF treatment Cbl was coimmunoprecipitated using an antibody against EGFR. As indicated in Figure 3D, knockdown of GnT-Va had a little effect on EGF-induced recruitment of Cbl to the EGFR. Tyrosine phosphorylation is required for the ubiquitin ligase activity of c-Cbl (Levkowitz et al. 1999). After c-Cbl pull-down using an anti-c-Cbl antibody, Western blotting with an anti-phospho-tyrosine antibody showed that phosphorylation of c-Cbl after EGF stimulation remained unchanged after knockdown of GnT-Va (Figure 3E). These results suggested that the inhibition of ligand-induced receptor downregulation caused by knockdown of GnT-Va was likely not due to defective c-Cbl-mediated ubiquitination of EGFR.

EGFR internalization and downstream signaling are impaired by knockdown of GnT-Va

The internalization of ubiquitinated EGFR into early endosomes is also a critical step for ligand-induced receptor downregulation (Di Fiore and Gill 1999). To detect whether abnormal internalization of EGFR accounted for impaired ligand-induced receptor downregulation, we next performed EGFR internalization experiments using several methodologies. Cells were starved and incubated with rhodamine-EGF or rhodamine-dextran at 37°C for 10 min and observed by immunofluorescence microscopy. As shown in Figure 4A, the internalized rhodamine-EGF was clearly observed in control cells and colocalized with early endosomes stained with an anti-EEA antibody. However, the internalization of rhodamine-EGF was significantly suppressed in GnT-Va knockdown cells. No difference in the internalization of rhodamine-dextran was observed between control and knockdown cells (data not shown), indicating the specificity of the effect of knockdown of GnT-Va on receptor internalization. Inhibited internalization of EGFR caused by knockdown of GnT-Va was further confirmed by measuring the EGF remaining...
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Fig. 3. Ligand-induced receptor dimerization, autophosphorylation, and ubiquitination are not affected by knockdown of GnT-V. (A) Cells were starved and stimulated with EGF (100 ng/mL) for 10 min. Cells were then crosslinked with BS3 at 4°C for 2 h, followed by lysis, SDS–PAGE, and Western blotting for EGFR. (B) Cells were starved and stimulated with EGF (100 ng/mL) for the indicated times. EGFR was immunoprecipitated (IP) with anti-EGFR followed by SDS–PAGE and Western blotting with antiphosphotyrosine (PY) and anti-EGFR antibodies (top panel). The graph shows the quantitation of PY levels from multiple experiments normalized to EGFR levels (bottom panel). (C) Cells were stimulated with EGF (100 ng/mL) for the indicated times, and cell lysates were prepared and subjected to SDS–PAGE and Western blotting for c-Cbl and ERK, respectively. (D) Cells were treated with EGF for different times, and EGFR was immunoprecipitated with an anti-EGFR antibody, followed by probing with anti-c-Cbl or EGFR antibodies (left panel). The graph shows the quantitation of c-Cbl levels from multiple experiments normalized to EGFR levels (right panel). (E) Cells were treated with EGF for the indicated times, and c-Cbl was immunoprecipitated with an anti-c-Cbl antibody, followed by SDS–PAGE and Western blotting with anti-PY and c-Cbl, respectively (left panel). The graph shows the quantitation of PY levels from multiple experiments normalized to c-Cbl levels (right panel). Each bar represents the mean (±S.D.) of triplicate determinations.

on the cell surface and receptor biotin labeling, respectively. After incubation with EGF at 37°C for up to 15 min, the remaining surface-bound EGF was stripped from the cell surface, TCA-precipitated, and detected with an antibody to EGF. The reduced cell surface EGF was observed after 10 min in control cells, indicating the internalization of the EGF/EGFR complex (Figure 4B). By contrast, the remaining EGFR on the cell surface of GnT-Vα knockdown cells was relatively unchanged after 15 min, suggesting an inhibition of the internalization of EGFR (Figure 4B). Similar results were obtained from the cell surface biotinylation experiment, where biotinylated EGFR on the cell surface was detected after the incubation of the cells with EGF for up to 15 min (Figure 4C).

Consistent with the observations in MDA-MB231 cells, knockdown of GnT-Vα in HT1080 cells also showed impaired ligand-induced EGFR internalization compared with control cells where EGFR, but not N-cadherin, was internalized 10 min after EGF stimulation, detected by cell surface biotinylation (Figure 4D), and further confirmed by immunofluorescent staining (data not shown).

Taken together, these results indicated that knockdown of GnT-Vα inhibited ligand-induced internalization of EGFR and decreased ligand-induced receptor downregulation due to the inhibition of EGFR internalization.

It is believed that ligand-induced receptor internalization and degradation play a critical role for regulating signaling intensity mediated by EGF (Clague and Urbe 2001). However, whether endocytosis leads only to the attenuation of EGFR-mediated signaling or is also important in triggering or amplifying EGFR-mediated signaling remains controversial (Di Fiore and Gill 1999; Ceresa and Schmid 2000; Di Fiore and De Camilli 2001). To test the effect of defective internalization of EGFR on EGF-induced signaling in GnT-Vα knockdown cells, EGF-induced ERK activation was determined by Western blotting using whole cell lysates. Concomitant with reduced ligand-induced receptor downregulation (Figure 4E, top panel), phosphorylation of ERK was inhibited in GnT-Vα knockdown cells at both short (5 min) and prolonged (18 h) treatment with EGF (Figure 4E), consistent with our previous report (Guo et al. 2007) indicating that impaired ligand-induced receptor internalization led to the attenuation of ERK signaling. This result is supportive of previous reports that the internalized, activated EGFR maintains its ability to generate and/or continue cell signaling after it is localized in early endosomes (Vieira et al. 1996; Shen et al. 2001; Sorkin and Von Zastrow 2002; Wang et al. 2002; Lua and Low 2005; Ren et al. 2008; Sigismund et al. 2008).

EGF-induced morphological changes, dephosphorylation of FAK, and SHP-2 activation were reversed by inhibiting receptor internalization of control cells

Knockdown of GnT-Vα by siRNA expression resulted in the significant inhibition of EGF-stimulated cell detachment from
EGFR internalization and downstream signaling are impaired by knockdown of GnT-V. (A) Cells were starved and incubated with rhodamine-labeled EGF (100 ng/mL) at 4°C for 1 h and then 37°C for 10 min. After the removal of an uninternalized cell surface ligand by an acid wash, cells were fixed and stained with an anti-EEA1 antibody. The location of EGFR (red), early endosomes (green), and nuclei (blue) were visualized by fluorescence microscopy. Arrows indicate colocalization of EGFR and early endosomes. Bar, 20 μM. (B) Cells were grown in 6-well plates, serum-starved for 2 h, and then incubated with serum-free media containing EGF (100 ng/mL) for 2 h at 4°C. After the removal of unbound EGF, cells were transferred to 37°C for the indicated times. At each time point, the remaining surface-bound EGF was stripped, concentrated by TCA-precipitation, and detected by Western blotting with an anti-EGF antibody after SDS–PAGE (left panel). As a control, the levels of ERK were determined in each monolayer after stripping of EGF. Images were quantitated from at least three independent experiments and expressed as percent EGF internalization representing the amount of EGF at each time point over the amount of EGF before the transfer of cells to 37°C (right panel). (C) Cells were serum-starved for 2 h at 37°C, and then incubated with serum-free media containing EGF (100 ng/mL) for 1 h at 4°C. Cells were transferred to 37°C and incubated for indicated times. Cell surfaces were biotinylated with NHS-LC-biotin, followed by streptavidin pull-down, SDS–PAGE, and probing with anti-EGFR. (D) HT1080 cells were serum-starved for 2 h at 37°C, and then incubated with serum-free media containing EGF (100 ng/mL) for 1 h at 4°C. Cells were transferred to 37°C and incubated for indicated times. Cell surfaces were biotinylated with NHS-LC-biotin, followed by streptavidin pull-down, SDS–PAGE, and probing with an anti-EGFR antibody or anti-N-cadherin antibody (left panel). Images were quantitated from at least three independent experiments and expressed as percent EGFR internalization representing the amount of EGFR at each time point over the amount of EGF before the transfer of cells to 37°C (right panel). (E) Cells were starved and stimulated with EGF (100 ng/mL) for the indicated times. Cell lysates were prepared and subjected to SDS–PAGE and Western blotting for indicated proteins.

Knockdown of GnT-Va also decreased EGF-mediated activation of the tyrosine phosphatase SHP-2, which, consequently, inhibited the EGFR-mediated dephosphorylation of focal adhesion kinase (FAK), consistent with the attenuation of invasiveness-related phenotypes that included decreased actin rearrangement and cell motility (Guo et al. 2007). If the inhibition of EGF-induced phenotypes resulted from decreased ligand-induced receptor internalization in GnT-Va knockdown cells, then the inhibition of EGF internalization of control cells should lead to the similar phenotypes as observed in GnT-Va knockdown cells (Guo et al. 2007). To this end, dansylcadaverine, an inhibitor of endocytosis, was used to treat cells (Sato et al. 2001). As shown in Figure 5A (left panel), ligand-induced EGFR internalization, detected by the cell surface biotinylation experiment, was significantly inhibited after pretreatment of cells with dansylcadaverine (500 μM) in control cells, indicating the inhibitory effect of dansylcadaverine on receptor endocytosis. As anticipated, EGF-induced refractile morphological changes and cell detachment were significantly inhibited in control cells, but not in GnT-V knockdown cells, by pretreatment of cells with dansylcadaverine (500 μM) (Figure 5A, right panel). Likewise, EGF-induced dephosphorylation of FAK was dramatically inhibited due to the inhibition of receptor internalization (Figure 5B), which was accompanied by the remarkable inhibition of SHP-2 activation induced by EGF following the pretreatment with the endocytosis inhibitor (Figure 5C). These results strongly support the conclusion that the altered EGF-mediated phenotypes of GnT-Va knockdown cells were results of impaired ligand-induced receptor internalization.

Altered internalization of EGFR in knockdown cells was not influenced by lactose treatment

Galectins are β-galactoside-binding proteins that bind to glycoproteins via their conserved carbohydrate recognition domains.
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Fig. 5. EGF-induced morphological changes, dephosphorylation of FAK, and SHP-2 activation were inhibited by inhibiting receptor internalization of MDA-MB231 control cells. (A) Cells were serum-starved for 1 h at 37°C, pretreated with or without dansylcadaverine (500 μM) for another 30 min at 37°C, and then incubated with serum-free media containing EGF (100 ng/mL) for 1 h at 4°C. Cells were transferred to 37°C and incubated for 10 min. Cell surfaces were biotinylated with NHS-LC-biotin, followed by streptavidin pull-down, SDS–PAGE, and probing with anti-EGFR (left panel). Cells were grown in 6-well plates and serum starved for 24 h, followed by pretreatment with or without dansylcadaverine (500 μM) for 30 min. Cells were then stimulated with EGF (100 ng/mL) for 10 min and observed by phase-contrast microscopy (right panel). (B) Cells were starved and pretreated with or without dansylcadaverine (500 μM) for 30 min, followed by stimulation with EGF (100 ng/mL) for the indicated times. Cell lysates were prepared for Western blotting for p-FAK and FAK after SDS–PAGE. (C) Cells were pretreated with or without dansylcadaverine (500 μM) for 30 min, and SHP-2 was immunoprecipitated for the SHP-2 activity assay after stimulation with EGF (100 ng/mL) for 10 min (top). The levels of precipitated SHP-2 detected by Western blotting were used for the normalization of SHP-2 activity (bottom). Each column represents the mean ± SD of activity from at least three independent experiments. Dan: dansylcadaverine.

(CRDs) (Barondes et al. 1994). Studies using galectin binding inhibitors have suggested that interactions between galectins and glycoproteins can generate a molecular lattice that can regulate the endocytosis of cell surface receptors and transporters (Partridge et al. 2004; Ohtsubo et al. 2005). To determine whether defective internalization of EGFR resulted from altered galectin-binding, we examined possible effects of GnT-Va knockdown on galectin expression and the binding of exogenous galectin to both cell lines. Galectin-3 and -1 are the major galectins expressed in MDA-MB 231 cells (unpublished data), and expression levels of both Gal-1 and Gal-3 were not affected by knockdown of GnT-Va, as shown in Figure 6A. Knockdown of GnT-Va caused reduction in exogenous binding of galectin binding, detected by biotinylated galectin, PE-labeled streptavidin, and flow cytometry (Figure 6B, left). EGF-treated knockdown cells also showed reduced galectin binding, but there was no difference compared to cells not treated with EGF (Figure 6B, right). No difference in endogenous cell surface galectin-3 was observed in control and knockdown cells before and after EGF treatment (Figure 6C). No significant changes in association of EGFR with galectin-1 and 3, determined by coimmunoprecipitation, were observed before and after EGF treatment (Figure 6D), suggesting that galectin-binding is not responsible for the inhibition of EGFR-induced internalization observed in GnT-Va knockdown cells. Moreover, we next tested cells with lactose, a competitive inhibitor of galectin binding, and, as a control, with sucrose to determine the effects on EGFR levels on the cell surface. The treatment of control cells with EGF after 10 min showed a decrease in cell surface EGFR, detected by cell surface receptor biotinylation (Figure 6E, left panel). Pretreatment of EGF-stimulated control cells with lactose, but not sucrose, resulted in more decreased levels of cell surface EGFR. By contrast, however, neither EGF nor lactose treatment had an effect on the levels of cell surface EGFR in GnT-Va knockdown cells (Figure 6E, left panel). Similar results were observed using HT1080 cells (Figure 6E, right panel), where the treatment of GnT-Va knockdown cells with lactose had no effect on EGFR internalization. These results using two cell types provide evidence that inhibited receptor-induced internalization of EGFR caused by GnT-Va knockdown was not likely due to galectin involvement.

Discussion

Our previous study using MDA-MB231 cells with GnT-Va siRNA expression showed that knockdown of GnT-Va suppressed EGF-induced ERK signaling, which caused inhibited activation of SHP-2 and dephosphorylation FAK, leading to aberrant invasiveness phenotypes with decreased cell detachment from matrix and cell motility (Guo et al. 2007). In addition to these short-time effects on signaling, the present study demonstrates that knockdown of GnT-Va also delayed the longer term
ligand-induced downregulation of EGFR. These effects resulting from reduced GnT-Va expression were due to changes in c-Cbl-mediated EGFR ubiquination and degradation, but rather a result of decreased receptor endocytosis and sustained cell surface residency. Moreover, pretreatment of control cells with an endocytosis inhibitor impeded EGF-induced activation of SHP-2 and dephosphorylation of FAK, mimicking the phenotypes observed in GnT-Va knockdown cells. Lastly, the effects of GnT-Va knockdown on receptor internalization were not mediated by altered galectin binding.

Ligand-induced receptor downregulation is an important biological process regulating the intensity and duration of receptor tyrosine kinase signaling (Di Fiore and Gill 1999; Clague and Urbe 2001). The proper downregulation of EGFR has been shown to be crucial in preventing cells from excessive EGFR-mediated downstream signaling and cellular transformation (Dikic and Giordano 2003; Marmor and Yarden 2004). Knockdown of GnT-Va by siRNA expression inhibited N-glycan expression on EGFR and attenuated EGF-induced ERK signaling without affecting its ligand binding (Guo et al. 2007). These results prompted us to further investigate the possible effects of inhibition of GnT-Va expression on receptor endocytosis. We found that suppression of GnT-Va impaired ligand-induced receptor downregulation of EGFR, resulting in increased receptor stability due to its delayed turnover. This effect of knockdown of GnT-Va on receptor downregulation could be mimicked by treatment of control cells with swainsonine, an inhibitor of Golgi α-mannosidase II or reversed by reintroduction of GnT-Va cDNA into knockdown cells, suggesting a direct involvement of GnT-Va modified N-glycans in regulating ligand-induced receptor downregulation.

The regulation of ligand-induced downregulation is a complex process involving many events (Grandal and Madshus 2008). First of all, EGFR, upon ligand stimulation, undergoes rapid dimerization, activation of intrinsic tyrosine kinase activity, and autophosphorylation of multityrosine residues of its intracellular domain. Although receptor dimerization and autophosphorylation contribute to the regulation of ligand-induced receptor downregulation (Wang et al. 2005; Padron et al. 2007), they were not affected by knockdown of GnT-Va, indicating that impaired ligand-induced receptor downregulation was not due to defective receptor dimerization or autophosphorylation. Once activated by ligand stimulation, one tyrosine phosphorylation residue (pY1045) of EGFR provides a direct binding site for c-Cbl, an ubiquitin-protein isopeptide ligase that mediates ligand-induced ubiquitination of EGFR (Joazeiro et al. 2005; Padron et al. 2007). After cells were transferred to 37°C and incubated for 10 min, surfaces were biotinylated with NHS-LC-biotin, followed by streptavidin pull-down and probing with anti-EGFR and galectin-3 antibodies following SDS–PAGE. As a control, total cell lysates were used for Western blotting for EGFR following SDS–PAGE.

Fig. 6. Inhibition of internalization of EGFR was not due to differential galectin binding. (A) Subconfluent cells were collected, lysed, and subjected to SDS–PAGE and Western blotting for galectin-1, galectin-3, and ERK (left panel); cell surfaces were biotinylated with NHS-LC-biotin, followed by streptavidin pull-down and probing with anti-galectin-1 and galectin-3 (right panel). LP: lectin precipitation. (B) Cells were serum-starved and stimulated with EGF (100 ng/mL) for 10 min. Cells were then collected and incubated with biotinylated galectin-1 (10 μg/mL) at 4°C for 30 min, followed by the flow cytometry assay using streptavidin–PE. (C) Cells were stimulated with EGF (100 ng/mL) for the indicated times, and cell surfaces were then biotinylated with NHS-LC-biotin, followed by streptavidin pull-down and probing with an anti-galectin-3 antibody after SDS–PAGE. (D) Cells were starved and stimulated with EGF (100 ng/mL) for 10 min. EGFR was immunopreciptated (IP) with anti-EGFR, followed by SDS–PAGE and Western blotting with anti-Gal-1, Gal-3, and EGFR. (E) MDA-MB231 (left panels) and GnT-Va knockdown HT1080 cells (right panel) were pretreated with lactose or sucrose for 24 h. Cells were serum-starved for 2 h at 37°C and incubated with EGF (100 ng/mL) for 1 h at 4°C with lactose or sucrose. After cells were transferred to 37°C and incubated for 10 min, surfaces were biotinylated with NHS-LC-biotin, followed by streptavidin pull-down and probing with anti-EGFR and galectin-3 antibodies following SDS–PAGE.
Receptor ubiquitination has been proposed to serve as a sorting signal which targets internalized receptor to clathrin-coated pits and lysosomes for degradation (Dikic and Giordano 2003; Marmor and Yarden 2004). Cbl-mediated receptor ubiquitination is a key regulator for ligand-induced receptor downregulation, but it may not be necessary for receptor internalization (Huang et al. 2006, 2007). In our study, we investigated c-Cbl-mediated ligand-induced degradation of EGFR and found that expression levels of c-Cbl, its phosphorylation levels, and association with EGFR were not affected before or after EGF stimulation of GnT-Va knockdown cells, indicating a normal function of c-Cbl in these cells. Therefore, defective c-Cbl-mediated EGFR degradation was not likely the cause of impaired ligand-induced downregulation of EGFR caused by depletion of GnT-Va.

Internalization of ubiquitinated EGFR into early endosomes is a prerequisite for efficient ligand-induced receptor downregulation. The accelerated internalization and efficient endosomal sorting of ubiquitinated EGFR to lysosomes lead to the pronounced downregulation of receptor (Di Fiore and Gill 1999; Grandal and Madshus 2008). In our study, we used three different methods to test whether knockdown of GnT-Va had an effect on ligand-induced receptor internalization. We showed that knockdown of GnT-Va caused significant inhibition of ligand-induced EGFR internalization. Reduced colocalization of EGFR with early endosomes was observed in GnT-Va knockdown cells, further confirming impaired receptor internalization in these cells. Therefore, defective EGFR internalization represented the most likely explanation for the observed impaired ligand-induced downregulation of EGFR due to knockdown of GnT-Va. Defective ligand-induced receptor downregulation was also observed when GnT-Va was knocked down in HT1080 cells. HT1080 cells are a human tumor cell line with the high expression of EGFR, and their tumor invasiveness-related phenotypes have been shown to be regulated by GnT-Va expression levels (Guo et al. 2003). Colocalization of EGFR with EEA1, an early endosomal marker, was similarly inhibited due to the depletion of GnT-Va in HT1080 cells, suggesting defective receptor internalization in these cells, similar to the breast carcinoma cells. Results from the HT1080 cells provided corroborating evidence that GnT-Va expression levels regulated ligand-induced receptor downregulation of EGFR by hindering internalization of activated receptor into early endosomes.

Following EGF stimulation, EGFR-mediated ERK signaling is activated. However, the effect of ligand-induced receptor endocytosis is the regulation of ERK signaling remains controversial. It is well established that endocytosis of activated EGFR from the cell surface to lysosomes leads to receptor degradation and attenuation of receptor signaling (Di Fiore and Gill 1999; Clague and Urbe 2001). On the other hand, there is an increasing body of evidence showing that the internalized EGFR remains highly phosphorylated on specific tyrosine residues and associated with Grb2 and Cbl (Sorkin and Carpenter 1991; Di Guglielmo et al. 1994), generating and amplifying ERK signaling from the endosomal compartment. The inhibition of ligand-induced endocytosis has been shown to block EGFR-mediated activation and the sustaining of ERK signaling (Vieira et al. 1996; Shen et al. 2001; Sorkin and Von Zastrow 2002; Wang et al. 2002; Pennock and Wang 2003; Lua and Low 2005; Ren et al. 2008), while the inhibition of recycling of receptor to the cell surface exhibited higher and sustained activation of ERK (Palmieri et al. 2006). A very recent study showed the inhibition of claritin-mediated endocytosis by knockdown of claritin dampened ERK signaling in HeLa cells (Sigismund et al. 2008). In the present study, we found that defective ligand-induced EGFR internalization was accompanied by inhibited phosphorylation of ERK, indicating an impaired ligand-induced activation of ERK signaling due to depletion of GnT-Va. This observation was consistent with the results in our previous report (Guo et al. 2007) and suggested that following knockdown of GnT-Va, the inhibition of ERK signaling may be attributed to decreased endosomal EGFR signaling caused by defective internalization of the activated receptor.

We showed that EGFR-induced cell detachment from matrix could be inhibited by treatment with a compound with dapsylcadaverine, an inhibitor that delays receptor internalization into early endosomes, hindering activation of SHP-2 and dephosphorylation of focal adhesion kinase. This inhibitory effect of dapsylcadaverine led to a cellular phenotype similar to that observed in GnT-Va knockdown cells (Guo et al. 2007), further supporting the conclusion that knockdown of GnT-Va resulted in defective ligand-induced internalization of EGFR and consequent inhibition of ERK signaling transduction.

Partridge et al. (2004) reported that in polyoma middle T-induced mouse mammary tumor cells in the GnT-Va null background a higher level of colocalization of EGFR was observed with EEA-1, an early endosomal marker, suggesting that the lack of GnT-Va expression increases EGFR endocytosis, a result that differs from those in the present study. Our results were based on the effects of the reduced expression of GnT-Va on ligand-induced EGFR endocytosis, while those of Partridge et al. were focused on nonligand-induced endocytosis, which is related to nonactivated EGFR without ligand stimulation. The effects of GnT-Va expression on EGFR endocytosis might, therefore, depend on receptor activation status. There may also be differences in how different tumor cell lines respond to decreased GnT-Va expression or between cells that have never expressed GnT-Va (null background) versus those cells whose GnT-Va expression was stimulated by oncogene stimulation and then inhibited by siRNA expression. The fact that both HT1080 cells, a fibrosarcoma line, and MDA-MB231, a breast carcinoma line, display reduced EGFR endocytosis when stimulated by EGF does demonstrate, however, that the effect is not limited only to the breast carcinoma line.

Recent studies suggest that galectins may form galectin–glycoprotein lattices on the cell surface which is believed to retain cytokine receptors and transporters on the cell surface and plays an important role in regulating receptor turnover and endocytosis (Partridge et al. 2004; Ohtsubo et al. 2005; Rabinovich et al. 2007; Ramasamy et al. 2007). Galectins represent a family of animal lectins with specific binding for β-galactosides (Barondes et al. 1994). Fifteen mammalian galectins have been identified so far and have been studied for their roles in regulating cell proliferation, apoptosis, and cancer progression (Breuer et al. 2002; Camby et al. 2006; Dümic et al. 2006). Partridge et al. (2004) suggested that glycan products of GnT-Va on EGFR and TGF-β are retained on the cell surface by galectin association, and their removal by constitutive endocytosis is retarded. In the GnT-Va null polyoma middle T-induced mammary tumor cells, greater colocalization of EGFR and EEA-1 was observed, indicating increased nonligand-induced endocytosis of EGFR in these cells compared to polyoma middle...
T-induced mammary tumor cells isolated from wild-type mice. When treated with lactose, a competitive inhibitor of galectin-3, the wild-type tumor cells showed greater colocalization of EGFR and EEA-1 (in the absence of exogenous EGF), suggesting that a lactose-inhibitable process was involved in the retention of EGFR on the cell surface. Another recent study using Gnt-T1Va null mice showed that the loss of Gnt-T1Va-modified N-glycans on pancreatic islet cells resulted in the attenuation of the glucose transporter 2 (Glut-2) cell surface half-life, increasing endocytosis with redistribution of the transporter into endosomes and lysosomes (Ohtsubo et al. 2005). Increased endocytosis of Glut-2 was likely the result of the decreased formation of galectin-9–Glut-2 complexes on the cell surface due to reduced Gnt-T1Va-modified N-glycans on Glut-2. N-Acetyllactosamine treatment decreased the cell surface localization of Glut-2 and inhibited galectin-9–Glut-2 complexes that were captured by chemical crosslinking. Pancreatic islet cells from Gnt-T1Va null mice showed no crosslinking of galectin-9–Glut-2, consistent with the conclusion that Gnt-T1Va glycosylation of Glut-2 and its interaction with galectin-9 regulated Glut-2 endocytosis. In the present study, the inhibition of ligand-induced EGFR internalization caused by knockdown of Gnt-Va did not appear to be directly galectin-mediated, however. First, an in vitro binding assay (Figure 6B) showed decreased binding of labeled, exogenously galectin-1 to Gnt-Va knockdown cells independent of EGFr-stimulation, consistent with the glycan products of Gnt-Va being cell surface ligands for galectin-1. If galectins regulated Gnt-Va-induced EGFR internalization, however, decreased cell surface galectin binding should result in increased receptor internalization, not decreased internalization, as we observed for two cell types. Second, expression levels of both galectin-1 and -3 and their association with EGFR before and after EGFR stimulation were not affected (Figure 6C and D). Third, the incubation of control cells with lactose for 24 h, the reported protocol for lactose treatment (Partridge et al. 2004), prior to EGFR stimulation did increase EGFR internalization, consistent with the results observed for wild-type polyoma middle T-induced mammary tumor cells. In Gnt-Va knockdown MDA-MB231 and HT1080 cells, however, this effect by lactose on EGFR-induced EGFR internalization was not observed, indicating that the inhibition of EGFR internalization caused by the reduction of Gnt-Va branching of glycans was not lactose-inhibitable.

Studies have shown that knockout or knockdown of Gnt-Va caused aberrant N-glycosylation of surface growth factor receptors and led, consequently, to altered signaling pathways mediated by these receptors (Partridge et al. 2004; Guo et al. 2005, 2007), which can result in aberrant gene expression. For example, knockout of Gnt-Va in mouse embryo fibroblasts caused the altered gene expression of other glycosyltransferases and membrane proteins such as β1 integrins (Guo et al. 2005) and galectins (Guo et al. 2008). Furthermore, the increased gene expression of integrins was also observed in SH-SY5Y cells after Gnt-Vb knockdown by siRNA (Abbott et al. 2006). A study using Gnt-Va null polyoma middle T-induced tumor cells (Lajoie et al. 2007) showed a reduction in the expression of caveolin-1, a coat protein of caveolae mediating endocytosis of some types of receptors. These findings show that the expression level of Gnt-Va can regulate the expression of not only N-glycans, but ultimately other cell surface proteins. In addition, studies have shown that several kinds of receptor tyrosine kinases (RTK), including EGFR, colocalize with cell adhesion molecules, such as integrins (Wu et al. 2004) and MUC1 (Ramasamy et al. 2007), on the cell surface and form clusters (Kotani et al. 2008). Altered N-glycosylation caused by knockdown of Gnt-Va could increase the clustering of EGFR with other surface molecules, due to either increased association among these molecules or increased expression of integrins and/or other surface proteins, resulting in defective ligand-induced receptor internalization of EGFR.

In conclusion, our results demonstrate that regulation of Gnt-Va expression affects the retention of EGFR on the cell surface after EGF stimulation, resulting in altered signaling pathways, including ERK and FAK phosphorylation. We clearly need to understand the mechanisms that cause this regulation and determine why in some cases lowered levels of N-glycan branching can result in decreased cell surface receptor retention, but, in the cases of MDA-MB231 and HT1080 cells, result in increased surface receptor retention.

Material and methods

Antibodies and chemicals

A polyclonal antibody against FAK, epidermal growth factor receptor (EGFR), EGF, ERK1/2, SH-PTP-2, monoclonal antiphosphotyrosine (PY20) and phospho-ERK, HRP-labeled anti-rabbit, and anti-mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies against EGFR and EEA1 were products of BD Biosciences (San Jose, CA). Anti-phospho-FAK was from Biosources. Anti-c-Cbl (7G10) was from Upstate Biotechnology (Billerica, MA). Mouse anti-ubiquitin and PE-streptavidin were from Invitrogen. Rhodamine-EGF, Alexa Fluor® 488 goat anti-rabbit, and anti-mouse IgG were products of Molecular Probes (Carlsbad, CA). EGF, swainsonine (SW), dansylcadaverine, and bovine serum albumin (BSA) were products of Sigma (St. Louise, MO), NHS-L-s-biotin and chemil crosslinker BS® were products of Pierce (Rockford, IL). Streptavidin–HRP was obtained from Rockland (Gilbertsville, PA). Biotinylated L-PHA and streptavidin–agarose were products of Vector Laboratories (Burlingame, CA). Biotinylated galectin-1 was generously donated by Dr Linda G. Baum (UCLA). The tyrosine phosphatase assay system was the product of Promega (Madison, WI).

Cells and cell culture

Human breast carcinoma cell lines MDA-MB231 and human fibrosarcoma HT1080 cells were from American Type Culture Collection (Manassas, VA). Both cell lines were cultured in complete Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (Hyclone) containing 10% FBS and 2 mM L-glutamine.

Transfection

Small interference RNA (siRNA) to human Gnt-Va was designed, and the pSUPER retroviral vector (pSUPER.retro.neo/gfp) with the insertion of Gnt-Va siRNA was produced as described (Guo et al. 2007). Cell transfections were performed with Lipofectamine™ 2000 according to the manufacturer’s instructions. Human pcDNA3/Gnt-Va plasmid was used for the transient expression of Gnt-Va. Cells were
incubated for 48–72 h after transfection, rinsed with cold PBS, and then lysed for the experiments (Guo et al. 2007).

**Galectin binding assay**

Serum-starved cells were stimulated with or without EGF (100 ng/mL) for 10 min and detached with 2 mM EDTA in PBS. Cells (10^6) were washed, resuspended in 100 μL of FCBr buffer (PBS containing 1% BSA and 0.01% sodium azide), and then incubated with biotinylated galectin-1 (10 μg/mL) at 4°C for 30 min, followed by incubation with phycoerythrin (PE)-conjugated streptavidin (10 μL) at 4°C for another 30 min. After wash with the ice-cold FCBr buffer, analysis was performed using the FACSCalibur (BD Biosciences) instrument.

**Western blotting, lectin blotting, and immunoprecipitation**

Subconfluent cells were harvested and lysed. Twenty micrograms of total cell lysates was electrophoresed on a 7% polyacrylamide mini gel, transferred onto a PVDF membrane, and then subjected to Western or lectin blotting (Guo et al. 2005, 2007). For immunoprecipitation, 500 μg total cell lysates protein were used and precipitates were then subjected to Western blotting, as described before (Guo et al. 2005, 2007).

**Cell surface labeling and immunoprecipitation**

Subconfluent cells were washed and detached using 2 mM EDTA. Cells were then washed twice with ice-cold PBS and incubated with 1 mg/mL NHS-LC-biotin in PBS for 20 min at 4°C on a rocking platform. After washing with PBS, cells were lysed and cell surface proteins were precipitated using 50 μL of streptavidin–agarose at 4°C overnight and detected by Western blotting as described before (Guo et al. 2002).

**Receptor degradation**

Cells were cultured in growth media containing 20 μg/mL cycloheximide and 100 ng/mL EGF for different times (Suyama et al. 2002). At each indicated time, cells were collected and EGFR was detected by immunoblotting with an anti-EGFR antibody. Immunoblots were scanned and the intensity of the protein bands was determined by densitometry.

**Receptor dimerization assay**

Cells were grown in 6-well plates to sub-confluency and serum-starved overnight. After incubated with or without 50 ng/mL EGF for 30 min at 4°C and then 5 min at 37°C, the cells were rinsed with ice-cold PBS and crosslinked by adding BS3 (final concentration 2.5 mM in PBS) on ice for 2 h. After 1 M Tris (pH 7.5) was added to a final concentration of 10 mM and incubated for another 15 min on ice, cells were then lysed with 1% NP-40 and EGFR dimerization was detected by immunoblotting after SDS–PAGE.

**EGF internalization assay**

Cells were grown in 6-well plates until sub-confluence, serum-starved for 2 h, and then incubated with serum-free media containing 100 ng/mL EGF for 2 h at 4°C. Cells were then washed with cold PBS to remove unbound EGF and transferred to 37°C for indicated time points. At each time point, the remaining surface-bound EGF was stripped from the cell surface using ice-cold 20 mM acetic acid/2 M NaCl for 5 min (Wong et al. 2002). The stripped EGF was TCA-precipitated and detected by immunoblotting after SDS–PAGE with an antibody to EGF. As control, the levels of ERK were determined in each monolayer after stripping of EGF. Data were expressed as the percent of the levels of EGF before the transfer of cells to 37°C.

**Internalization of EGFR and dextran**

The receptor internalization assay was performed as described with a minor modification (Sato et al. 2001). In brief, cells were grown to sub-confluency in chamber slides and then serum-starved for 2 h at 37°C. Cells were transferred to 4°C and incubated in serum-free media containing rhodamine-conjugated EGF (100 ng/mL) for 1 h at 4°C. Cells were then switched to 37°C and incubated for 10 min. Cells were put back on ice and cell surface ligand stripped with ice-cold 0.2 M acetic acid (pH 2.5) that contained 0.5 M NaCl for 5 min. After washing with PBS, cells were fixed in HistoChoice for 15 min at room temperature and permeabilized with cold methanol at −20°C for 3 min. After blocking with 10% goat serum, cells were stained with anti-EEA1, followed by incubation with Alexa Fluor® 488 goat anti-mouse IgG (1:250). After washing with PBS, the chamber slides were mounted and the cells subjected to deconvolution fluorescence microscopy. Nuclei were visualized with 1:10,000 dilution of Hoechest 231(Molecular Probes).

**Endocytosis analysis by cell-surface biotin labeling**

EGFR endocytosis was analyzed using cell surface biotinylation as reported (Tanos and Pendergast 2006). In brief, cells were grown to sub-confluency in 100 mm culture dishes, serum-starved for 2 h at 37°C, and then incubated with serum-free media containing 100 ng/mL EGF for 1 h at 4°C. Cells were transferred to 37°C and incubated for indicated times. EGFR internalization was stopped by placing the cells at 4°C. Cells were then washed twice with ice-cold PBS and incubated with 1 mg/mL NHS-LC-biotin in PBS for 20 min at 4°C on a rocking platform. After washing with PBS, cells were lysed and biotinylated as described above. The pellets were washed and subjected to SDS–PAGE. The gels were then transferred onto a PVDF membrane and probed with an antibody against EGFR.

**Tyrosine phosphatase activity assay**

Subconfluent cells were starved in serum-free media overnight and then stimulated with EGF (100 ng/mL) for 10 min. For some experiments, cells were pretreated with dansylcadaverine (500 μM) for 30 min at 37°C before subjected to EGF stimulation. After cells were lysed in the ice-cold buffer, phosphatase SHP-2 was immunoprecipitated and precipitated proteins were then subjected to an in vitro activity assay performed with a tyrosine phosphatase assay kit (Guo et al. 2007). The levels of precipitated SHP-2 were detected by Western blotting and used for the normalization of SHP-2 phosphatase activity.

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References

Abbreviations

BSA, bovine serum albumin; CRDs, carbohydrate recognition domains; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FBS, fetal bovine serum; GnT-V, N-acetylglucosaminyltransferase V; L-PHA, leucogluclusinating phytohemagglutinin; MES, 4-morpholineethanesulfonic acid; PBS, phosphate-buffered saline; PE, phycoerythrin; PTP, protein-tyrosine phosphatase; SHP-2, Src homology 2 domain-containing PTP; siRNA, small interfering RNA.

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Conflict of interest statement

None declared.

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