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<td>学位授与の日付</td>
<td>平成28年3月10日</td>
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<td>学位授与の要件</td>
<td>学位規則第4条1項該当</td>
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<td>学位論文題名</td>
<td>Integrin α5 suppresses the phosphorylation of EGFR and its cellular signaling of cell proliferation via N-glycosylation</td>
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Integrin α5 suppresses the phosphorylation of EGFR and its cellular signaling of cell proliferation via N-glycosylation

「論文内容要旨」
Integrin α5 suppresses the phosphorylation of EGFR and its cellular signaling of cell proliferation via N-glycosylation

Integrins are the αβ heterodimer membrane proteins that consist of the α subunits and the β subunits. As the major adhesion molecular, integrin-mediated cell adhesion can regulates a multitude of cellular responses, including cell migration, proliferation, survival, and cross-talk between other cell membrane receptors. Until now, 24 different kinds of integrins have been reported, during which the function of integrin α5β1, a major fibronectin receptor, is most famous and well-established. Therefore, our research is focused on the integrin α5β1.

Integrin α5β1, an N-glycosylated protein, is essential for many biological functions. The N-glycosylation of integrin α5β1 is thought to play crucial roles in cell spreading, migration, ligand binding, and dimer formation, but the detail mechanisms remain unclear. Integrin α5 contains 14 potential N-glycosylation sites, during which our laboratory have previously identified that three potential N-glycosylation sites, No. 3, 4, and 5, of it are essential for the α5β1 heterodimer formation, cell surface expression, and cell adhesion (Isaji, T., et al., JBC, 281,33258, 2006). However, the functions of the other remainder N-glycosylation sites are still unknown.

In response to fibronectin-mediated cell adhesion, integrin α5β1 not only directly initiate certain cytoplasmic signals for cell spreading, but also indirectly modulate the transmission of several other membrane receptor signalings, which are referred to as the cross-talks, especially the epidermal growth factor receptor (EGFR) signaling. The cross-talk between integrin α5β1 and EGFR can also controls many cell behaviors, especially cell proliferation. However, the function of N-glycosylation on integrin α5 in integrin α5β1/EGFR cross-talk-mediated cell proliferation and cellular signaling remains unclear. In addition, there have been several controversial reports about the regulation of EGFR-mediated signaling by integrin α5 subunit.

As we know, EGFR, a member of the ErbB receptor tyrosine kinase family, converts extracellular cues into intracellular effectors, triggering appropriate cellular responses, which is important for normal epithelial developmental biology and in cancer metastasis. A dysregulation of EGFR signaling, including receptor overexpression and/or activation, is a common feature in tumorigenesis. Due to this aberrant activity in the pathology of tumor, EGFR has emerged as an attractive candidate target for anticancer therapy, which prompted us to examine the underlying molecular mechanisms for EGFR signal activation. EGFR signal activation forms a complex signaling network with several regulators, including its related-cytoplasmic proteins, microRNAs, tyrosine kinase inhibitors, and other coupled (or cross-talked)-membrane receptors. However, studies that address these direct or indirect
regulations of EGFR activation have focused mainly on the inner membrane, particularly the cytoplasmic kinase domain of EGFR. The outer membrane underlying mechanisms remain unknown.

Current insight into this regulation derives largely from studies around EGFR-related microdomains, also called lipid rafts, which are thought to act as platforms for EGFR signal transduction. The cell membrane microdomains are usually rich in cholesterol, glycosphingolipids (GSLs), and some membrane glycoproteins. Several studies have associated GSLs, including gangliosides GM1, GM3, and GD3, with the regulation of EGFR signal activation. In addition to GSLs, some glycoproteins that are located in the EGFR-related microdomains, play important roles in the regulation of EGFR signaling. These limited results highlight the possibility that glycosylation might act as a ‘linker’ in lipid rafts for the regulation of EGFR signaling. However, little is known about how glycosylation controls this kind of regulation. Therefore, exploring the underlying mechanisms involved in the glycosylation-mediated regulation of EGFR signaling, is very important for a complete view of the biological functions of EGFR.

Interestingly, some integrins, including integrin α5, serve as important members of the EGFR lipid rafts-related glycoproteins, as mentioned above. And the integrins mediate cooperation with EGFR mainly through α-cytoplasmic domains, which are also restricted to the inner membrane. Therefore, how the N-glycans of integrin α5 subunit regulate EGFR function is becoming an attractive topic.

Taken together, in order to resolve these issues, we have examined the relationship between integrin α5 subunit and EGFR in this thesis, and found that the integrin α5 can negatively regulates EGFR-mediated cellular signaling and cell proliferation. We used N-glycosylation mutants of integrin α5 to clarify the roles of the N-glycosylation of α5 subunit in EGFR-mediated signaling and cross-talk with EGFR, and found that the regulation was strictly controlled via the N-glycans of integrin α5 subunit, particularly the N-glycan on potential N-glycosylation site 11. The specific experimental procedures and results are as follows:

1. First, we established a simple cell model to clarify the underlying molecular mechanism for the cooperation between integrin α5 and EGFR. The human EGFR cDNA was transfected into the CHO-B2 cell line that lacks the α5 subunit, in order to establish a stable EGFR-overexpressed CHO-B2 cell line (CHO-B2/EGFR). The CHO-B2/EGFR cells then were respectively reconstituted with either a GFP-tagged wild-type (WT) or a N-glycosylation mutant α5 subunit, S3-5, either of which only contains three of the 14 N-glycosylation sites. Unexpectedly, the expression of WT, but not S3-5 α5 subunits, significantly suppressed the cell proliferation, colony formation and tumor formation compared with those found in control cells with or without overexpression of a GFP tag only. Taken together, these results suggested that the remaining N-glycosylation sites, with
the noted exception sites 3-5 of integrin α5 play an important role in the inhibition of cell growth and tumorigenecity.

2. Considering the overexpression of EGFR in these cells, we compared the EGFR-mediated cellular signaling in these cells. Surprisingly, under normal culture conditions, the phosphorylation levels of EGFR and its downstream molecules ERK and AKT were significantly decreased in WT cells compared with those in other cells. Furthermore, the responses for exogenous EGF-induced cell proliferation and cellular signaling were also significantly suppressed in WT cells, but neither S3-5 cells nor in the other control cells. These results supported our hypothesis that integrin α5 inhibits EGFR-mediated signaling via N-glycosylation.

3. Next, we selected several human cancer cell lines, such as HeLa, U-251MG, and MDA-MB-231 cells, which express relative high levels of endogenous EGFR, in order to confirm whether the phenomenon is common to other mammalian cells. By using a CRISPR/Cas9 knockout system, the inhibitory effects of integrin α5 on cell growth and EGFR cellular signaling are also observed in these human cancer cell lines. Together, these results further suggest that integrin α5 is a negative regulator for EGFR-mediated signaling through N-glycosylation.

4. Upon EGF binding, EGFR is involved in a series of trafficking events, including internalization, degradation, and recycling, which ultimately regulate its signal amplification and propagation. We compared the EGFR endocytosis between these cell lines, and found that the expression of integrin α5 delays EGFR internalization upon EGF stimulation. These results clearly indicated that EGFR internalization was delayed by α5 via N-glycosylation, which might explain why the EGFR signaling was inhibited in the WT cells.

5. Given the evidence that the N-glycosylation of integrin α5 regulated EGFR internalization and activation. Therefore, we wondered how N-glycosylation participates in regulation, and whether integrin α5 is associated with EGFR through the N-glycosylation of α5. By reciprocal immunoprecipitation and OptiPrep density gradient ultracentrifugation experiments, we identified that the N-glycosylation of α5 plays a crucial role in its localization and complex formation with other receptors on the cell membrane.

6. The data provided above led us to seek the N-glycosylation site(s) of α5 that was essential for its growth inhibitory effect. Therefore, we focused on the N-glycosylation of the calf domain, which happens in the vicinity of the cell membrane. Deletion of the
"N-glycosylation sites on the calf domain of α5 abolished its inhibitory effects on cell growth. Furthermore, recently, we identified that the No. 11 N-glycan on calf domain was most important for the inhibition, demonstrating this N-glycosylation of integrin α5 plays a crucial role in the regulation of EGFR-mediated cell signaling, which demonstrates a novel regulator for EGFR inhibition.

Taken together, we described here how cell growth could be downregulated by integrin α5, and clearly demonstrated how the N-glycosylation of α5 is a key factor for regulation. Among 14 potential N-glycosylation sites of α5, site 11 on the calf domain played a crucial role in the inhibitory effect on EGFR-mediated signaling, through regulation of the complex formation between EGFR and α5, localization in lipid rafts, and internalization (Figure). Thus, this thesis outlines the novel underlying mechanism responsible for the inhibition of EGFR, and also provides new insight into the role of N-glycosylation in the regulation of cellular signaling to maintain cell properties via a cross-talk manner among glycoproteins on the cell surface.

Reference: The Main Thesis (Original Article)