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Synergistic inhibition of cell migration by tetraspanin CD82 and gangliosides occurs *via* the EGFR or cMet-activated Pl3K/Akt signalling pathway

Ying Li[b,1], Xiaohua Huang[a,c,1], Jianing Zhang[a], Yuzhong Li[b], Keli Ma[a,*]

[a] Department of Biochemistry and Molecular Biology, Dalian Medical University, Dalian 116044, China
[b] Department of Clinical Laboratory, Second Affiliated Hospital of Dalian Medical University, Dalian 116023, China
[c] Department of Clinical Biochemistry, College of Laboratory Medicine, Dalian Medical University, Dalian 116044, China

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ABSTRACT

The metastasis suppressor CD82/KAI-1, which is a member of the tetraspanin superfamily, has been proposed to exert its activity together with glycosphingolipids. However, the mechanism of CD82 inhibition has not been fully elucidated. The present study aimed to investigate the synergistic inhibition of cell migration by the tetraspanin CD82 and gangliosides and to correlate this inhibition with activation of epidermal growth factor receptor (EGFR) and hepatocyte growth factor receptor (HGFR/cMet) in Hepa1-6 cell lines, whose motility and migration is stimulated by epidermal growth factor (EGF) and hepatocyte growth factor (HGF) *in vitro*. We found that Hepa1-6 cells transfected with the CD82 gene exhibited decreased migration in response to EGF and HGF. EGF-stimulated phosphorylation of EGFR at Tyr1173 was inhibited in these cells, which contributed to the attenuation of EGFR. Ectopic expression of CD82 in Hepa1-6 cells inhibited HGF-stimulated tyrosine phosphorylation of cMet at Tyr1313 and Tyr1365 without affecting the expression of cMet. These inhibitory effects were enhanced when CD82 was introduced with Ganglioside GM3 alone or GM2/GM3. Reduction of CD82 expression by RNA interference together with depletion of glycosphingolipids with P4 significantly enhanced cell motility and increased the expression of EGFR and its phosphorylation at Tyr1173 in response to EGF. Increased cell motility and HGF-dependent activation of cMet at Tyr1313 and Tyr1365 resulted from decreased CD82 levels and increased GM3. Furthermore, CD82 expression selectively attenuated EGFR and cMet signalling *via* phosphatidylinositol 3-kinase/Akt but had no affect on the activity of the MAPK signalling pathway. These results suggest that the synergistic effects of CD82 and GM3 or GM2/GM3 on EGFR expression and phosphorylation and cMet activation are responsible for CD82 inhibition of EGF- and HGF-dependent cell motility and migration of Hepa1-6 cells.

1. Introduction

CD82/KAI-1 is a metastasis suppressor and a member of the tetraspanin superfamily of transmembrane proteins that is widely distributed in normal tissues. Previous *in vitro* work has shown that ectopic expression of CD82 suppresses tumour cell migration, which is a process underlying the dissemination of tumour cells *in vivo* (Nagira et al., 1994; Takaoka et al., 1998). The function of CD82 is not fully understood, and it has been proposed that association of CD82 with other cell surface components may be crucial for its biological activities (Hemler et al., 1996; Maecker et al., 1997). Glycosphingolipids (GSLs) at the cell surface membrane are associated with tetraspanins, growth factor receptors, signal transducers, and integrins. This organisational framework is termed the "glycosynapse" and is important for GSL-modulated or GSL-dependent cell adhesion, motility, and growth (Hakomori and Handa, 2002; Hakomori, 2004). Tetraspanins have been reported to play a critical role in regulating receptor tyrosine kinase signalling. CD82 has been shown to affect signalling mediated by at least 2 receptor kinases present in nonimmune cells, EGFR and cMet. The CD82 tetraspanin is directly associated with the EGF receptor (EGFR), and ectopic expression of CD82 in epithelial cells suppresses EGF-induced lamellipodial extensions and cell migration (Odintsova et al., 2000). Previous studies have also reported that CD82 regulates cell processes that are mediated by cMet. GM2 and the TSP CD82 exert inhibitory effects on hepatocyte growth factor (HGF)-induced activation of cMet and its cross-talk with integrin $\alpha 3\beta 1$. The GM2 and GM3 heterodimeric ganglioside complex is closely

* Corresponding author. Tel.: +86 411 86110311; fax: +86 411 86110309; mobile: +86 15998663363.
E-mail address: makelipaper@163.com (K. Ma).
[1] These authors contributed equally to this work.

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associated with the cMet-CD82 complex that negatively regulates HGF-dependent cMet activation and cell motility (Todeschini and Hakomori, 2008; Todeschini et al., 2008). The ability of CD82 to modulate RTK signalling may be mediated by gangliosides, which are found within tetraspanin-enriched microdomains (Park et al., 2009; Murayama et al., 2008).

To elucidate the molecular mechanism by which the tetraspanin CD82 and gangliosides synergistically affect EGFR and cMet signalling, we examined the tyrosine kinase activity of EGFR and cMet, Akt and MAPK activation, and cell motility and migration of Hepa1-6 cell lines *in vitro* after down-regulation or up-regulation of CD82 expression. These activities were also analysed following P4 pretreatment, which depletes all GSLs, as well as in the presence or absence of exogenous GM3 addition.

2. Materials and methods

2.1. Antibodies and reagents

2.1.1. Antibodies

Anti-p-EGFR (Tyr1173) IgG, anti-EGFR IgG, anti-p-Met (Tyr1313) IgG, anti-p-Met (Tyr1365) IgG, anti-Met IgG, anti-Akt1/2/3 IgG, anti-p-Akt1/2/3 (Ser473) IgG, anti-p-Akt1/2/3 (Thr308) IgG, anti-Erk1/2 IgG, anti-p-p44/42MAPK (Erk1/2) (Thr202/Tyr204) IgG, anti-β-actin mouse IgG, goat anti-rabbit IgG-horseradish peroxidase, rabbit anti-goat IgG-horseradish peroxidase, goat anti-mouse IgG-horseradish peroxidase and horseradish peroxidase-labelled streptavidin were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-V5 IgG was purchased from Invitrogen (Carlsbad, CA). Anti-GM3 IgM was obtained from Wako Pure Chemical Industries Ltd.

2.1.2. Reagents

EGF was obtained from ProSpec-Tany TechnoGene Ltd.; HGF was purchased from Genentech (San Francisco, CA); Lipofectamine™ 2000 and opti-MEM were obtained from Invitrogen (Carlsbad, CA); protease inhibitor cocktail was obtained from Sigma–Aldrich (St. Louis, MO); D-threo-1-phenyl-2-palmitoyl amino-3-pyrrolidino-1-propanol (P4) was purchased from Matreya, Inc. (Pleasant Gap, PA); GM3 and GM2 was purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture

Mouse hepatocellular carcinoma Hepa1-6 cells were grown in 10-cm cell culture dishes or in multi-well plates in 1640 medium supplemented with 10% heat-inactivated FBS,100 units/mL penicillin, and 100 mg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere.

2.3. RNAi knockdown of CD82 expression in Hepa1-6 cells

A negative control and four CD82 shRNA plasmids were constructed.

They were PGPU6/GFP/Neo-shNC, PGPU6/GFP/Neo-CD82-mus-251, PGPU6/GFP/Neo-CD82-mus-557, PGPU6/GFP/Neo-CD82-mus-604, and PGPU6/GFP/Neo-CD82-mus-788. To identify an effective shRNA plasmid that inhibited CD82, Hepa1-6 cells were transfected with the CD82 shRNA plasmids using Lipofectamine™ 2000 in opti-MEM medium. The most effective CD82 shRNA plasmid was selected following RT-PCR analysis. Total RNA was isolated from cells using Trizol (Invitrogen), and reverse transcription was carried out using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Relative quantitative analysis consisted of normalising CD82 levels to the levels of the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The mouse CD82 forward primer was 5′-GGGCCCTCTTCTACTTCAACAT-3′, and its reverse primer was 5′-AGAAAGGCTGTTGTCCTCTTCC-3′. The mouse GAPDH forward primer was 5′-TACTTATGCCGATGTCGTTGT-3′, and its reverse primer was 5′-CCAGCCTCGTCCCGTAGA-3′. Two days after transfection, CD82 expression was measured by Western blot. The PGPU6/GFP/Neo-CD82-mus-604 displayed the most effective inhibition and was used for additional experiments in this study.

2.4. Up-regulation of CD82 by transfection of the CD82 gene into Hepa1-6 cells

The CD82 gene was cloned into the pcDNA4.0V5 vector and transfected into Hepa1-6 cells using Lipofectamine™ 2000 in opti-MEM medium. Two days after transfection, CD82 expression was measured by Western blot using an anti-V5 antibody.

2.5. Analysis of ganglioside expression in Hepa1-6 cell by HPTLC and flow cytometry analysis

To decrease ganglioside expression in Hepa1-6 cells, cells were treated with P4, a specific inhibitor of UDP-glucose ceramide glucosyltransferase (a key enzyme in the synthesis of glucosylceramide). For P4 treatment, cells were seeded into 12-well plates in 1640 medium supplemented with 10% FBS and 1.0 μM of P4. After a 48-h incubation, the cells were harvested and the inhibition of ganglioside synthesis was monitored by HPTLC. HPTLC analysis was performed as described previously (Ladisch and Gillard, 1985). Briefly, cells were grown in 10-cm dishes until ~90% confluent. The cells were trypsinised and washed three times with PBS. Total lipids were extracted twice from the cell pellet with chloroform/methanol (1:1 by volume). The extracts were combined and dried under a stream of N_2. The gangliosides were purified by partitioning the dried total lipid in di-isopropyl ether/1-butanol/17 mM aqueous NaCl followed by Sephadex G-50 gel filtration and lyophilisation. Individual gangliosides were separated on silica gel 60 HPTLC plates (Merck, Darmstadt) with a solvent system of chloroform/methanol/0.25% aqueous $CaCl_2 \cdot 2H_2O$ (60:40:9 by volume). The gangliosides were visualised as purple bands by spraying the plates with resorcinol–HCl reagent and heating to 120 °C.

To increase ganglioside expression in Hepa1-6 cell, cells were treated with 50 μM GM3 for 48 h before harvesting. Cells were detached by trypsin/EDTA and washed with PBS. Aliquots of cells (1×10^5) were incubated with mouse anti-GM3 IgG for 1 h on ice, washed with PBS, incubated with goat anti mouse FITC-IgM for 40 min on ice, fixed in 2% paraformaldehyde/PBS, and analysed using a Coulter EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA).

2.6. In vitro cell migration assay

Cell migration assays were performed using both scratch wound analysis and chemotaxis migration assays (Boyden chamber, Costar, Cambridge, MA, USA) with 8 μm pore polycarbonate filters (BD Biosciences, Franklin Lakes, NJ). Cell migration analysis was performed with Hepa1-6 cells that were pretreated with P4 or GM3 and transfected with the CD82 shRNA plasmid or the CD82 gene as described above. After the cells were incubated in serum-free medium overnight, a scratch was made. The cells were washed 3 times with PBS and incubated in serum-free medium containing 50 ng/mL EGF or 100 ng/mL HGF. Plates were photographed at 200× magnification after 24 h.

Chemotaxis migration assays were performed with Hepa1-6 cells that were pretreated with P4 or GM3 and transfected with the CD82 shRNA plasmid or the CD82 gene as described above. After the cells were incubated in serum-free medium overnight, the cells (1×10^5) were resuspended in 300 μL of serum-free medium