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Expression of Folate Receptor Protein in CHO Cell Line

Chong Ho Kim[1,†] and Seung-Taeck Park[2]

[1]*Department of Clinical Laboratory Science, Wonkwang Health Science College, Iksan 570-750, Korea.*
[2]*Department of Anatomy, College of Medicine, Wonkwang University, Iksan 570-749, Korea*

One of cell surface receptor proteins, human folate receptor (hFR) involves in the uptake of folates through cell membrane into cytoplasm, and is anchored to the plasma membrane by a fatty acid linkage, which has been identified in some cells as a glycosylphosphatidylinositol (GPI)-tailed protein with a molecular mass of about 40 kDa. The hFR is released by phosphatidylinositol phospholipase C (PI-PLC) because it contains fatty acids and inositol on the GPI tail. Caveolin decorates the cytoplasmic surface of caveolae and has been proposed to have a structural role in maintaining caveolae. It is unknown whether caveolin is involved in targeting, and is necessary for the function of GPI-tailed proteins. To compare the ability of folic acid binding, internalization and expression of hFR, and the effect of caveolin at the both apical and basolateral side of cell surfaces in Chinese hamster ovary (CHO) clone cells overexpressed the hFR and/or caveolin. Our present results suggest a possibility that the overexpression of caveolin does not be involved in expression of hFR, but plays a role as a factor in PI-PLC releasing kinetics, and for a regulation of formation, processing and function of hFR in CHO clone cells overexpressed caveolin.

Key Words: Human folate receptor, CHO, GPI tailed protein

INTRODUCTION

The folates are important sources for DNA synthesis and essential vitamins for cell growth. It has been reported that a membrane receptor protein, a high affinity folate-binding protein involves in the uptake of folates through cell membrane into cytoplasm (Antony, 1992; Figini et al., 2003; Ratnam et al., 2003). One of cell surface receptor proteins, human folate receptor (hFR) is anchored to the plasma membrane by a fatty acid linkage, which has been identified in some cells as a glycosylphosphatidylinositol (GPI)-tailed protein with a molecular mass of about 40 kDa (Luhrs and Slomiany, 1989). The hFR in some normal tissues has been identified by use of immunological method (Kamen and Capdevila, 1986; Antony, 1996; Wu et al., 1999; Matherly, 2001), KB cells (Elwood, 1989). It was also identified in body fluids (Antony, 1992). cDNA for hFR has been cloned from human KB cells (Elwood, 1989; Sadasivan and Rothenberg, 1989), human colon carcinoma cells (Lacey et al., 1989), human ovarian carcinoma cells (Buist et al., 1995), human placenta cells (Green and Ford, 1984). Although their 5'-untranslated regions vary from one to another, all the cDNAs derived from human cancer cell lines have the same coding sequences (Coney et al., 1991). Researchers demonstrate that the hFR expressed on the membranes of MA 104 money kidney cells is released from the plasma membrane by phosphatidylinositol phospholipase C (PI-PLC) treatment because the hFR contains fatty acids and inositol on the GPI tail (Lacey et al., 1989; Sinn et al., 2003).

Caveolae has a coat that includes caveolin, as well as other signaling molecules, such as heterotrimeric guanosin triphosphatase (GTPase), tyrosine kinases, and calcium pumps, Ca-ATPase, and GPI-anchored proteins are associated with caveolae (Bagnoli et al., 2000; Park et al., 2001: McFarland et al., 2004). Caveolae function has been reported to be regulated by kinase activity (Parton et al., 1994). Caveolin is a 22 kDa tyrosine phosphoprotein that is enriched within the caveolae (Schnitzer et al., 1995; Engelman et al.,

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†Corresponding author: Chong-Ho Kim, Department of Clinical Laboratory Science, Wonkwang Health Science College, Iksan 570-750, Korea.
Tel: 82-63-840-1213, Fax: 82-63-840-1219
e-mail: chkim@wkhc.ac.kr

1999; Fujimoto et al., 2000; Nohe et al., 2005). Caveolin decorates the cytoplasmic surface of caveolae and has been proposed to have a structural role in maintaining caveolae. It is unknown whether caveolin is involved in targeting GPI-tailed proteins to the plasma membrane, or whether caveolin is necessary for the function of GPI-tailed proteins. Based on the activity of the many proteins associated with caveolae, these structures have been implicated in signal transduction, calcium regulation, and nonclathrin-dependent receptor-mediated transport processes such as transcytosis and endocytosis (Scherer et al., 1994; Kim et al., 1999; Shu et al., 2000; Pol et al., 2005). However, the exact function of caveolae has not been determined.

In this paper, we investigated the cell surface folic acid binding activity and internalization of folate on the both apical and basolateral surfaces in CHO clone cells. We also determined whether caveolin expression is a determinant of hFR function.

MATERIALS AND METHODS

1. Materials

dl-N-5-methyltetrahydrofolate ([^{3}H]5-MTHF) and [^{3}H]-folic acid was purchased from Moravek Biochemicals (Brea) and [^{125}I]-labeled pteroylglutamic acid ([^{125}I]-folic acid)was purchased form New England Nuclear (Boston). Agarose was from Bethesda Research Laboratories (Gaithersburg). All restriction enzymes, DNA modifying enzymes, plasmids and *E. coli* were purchased from Promega (Madison). All other reagents were of reagent grade or higher, and were purchased from Sigma Chemical Co. (St. Louis), Fisher Scientific (Pittsburgh), or Baker Inc. (Phillipsburg).

2. Plasmid construction of hFR and caveolin

The pRc/CMV vector (Invitrogen) contains a cytomegalovirus (CMV) promoter and a neomycin resistance gene. The cDNA encoding human folate receptor (hFR) was isolated from a placental cDNA library and subcloned at the *EcoR* I multiple cloning site of pGEM4Z (Promega). The *Hind* III-linearized pRc/CMV expression vector was ligated with blunt-ended hFR cDNA insert using T4 DNA ligase (Promega). Competent *E. coli* (JM109, Promega) were transformed with the ligation mixture. Recombinant plasmids were isolated by the Qiagen (Gaithersburg) maxiprep kit.

We designated the plasmid construct containing the pRc/CMV vector and the hFR cDNA as pRc/FR. The caveolin mRNA isolated from human lung (Clontech) was reverse transcribed using the first strand kit (Perkin Elmer). The caveolin cDNA was amplified with the 5' sense primer corresponded to bp 13-34 and the 3' antisense primer corresponded to bp 931~905 of caveolin sequence including *Hind* III and *Xba* I sites. The caveolin cDNA was subcloned into the *Hind* III and *Xba* I sites within the pRc/CMV vector. We designated the plasmid construct as pRc/CA. The folate receptor cDNA and caveolin cDNA in pRc/CMV vector were verified by restriction enzyme digests and by DNA sequencing using ^{35}S-dATP (Amersham).

3. Stable transfection

For stable transformants of CHO cells, CHO cells were transfected with 10 g of pRc/FR and/or pRc/CA by using the electroporation method (BioRad) with 4×10^6 cells in 0.4 ml PBS, at 300 Volt and 960 mF settings. Stable transfectants were selected in 250 mg/ml G418 and were maintained in 125 mg/ml G418. We designated the stable clone transfected with pRc/FR alone as CloneA, with pRc/CA alone as Clone B, and with both pRc/FR and pRc/CA as Clone AB.

4. Tissue culture

CHO cells for our experiments were cultured in deplete minimal essential media (9/10DMEM) with a final folic acid concentration of approximately 200 nM. Cells were grown in monolayers in 10 cm tissue culture dishes containing 10 ml media at 37℃ under a humidified atmosphere of 5% CO_2, and were subcultured weekly. To harvest the cells, the medium was decanted, and 1~2 ml of 2.5% trypsin solution was added to the dishes. The cells were incubated at room temperature for 2 min, the excess solution was decanted, and the cells were immediately resuspended in media. The following reagents, replete minimal essential media (RMEM) containing 2 μM folic acid with Earle's salts and L-glutamine, deplete minimal essential media (DMEM) without added folic acid with Earle's salts and L-glutamine, trypsin solution

(2.5%), penicillin/streptomycin/fungizone (PSF) solution (100X), fetal calf serum (FCS) and geneticin sulfate (antibiotic G418) were purchased through Gibco Laboratories (Grand Island).

5. Western blot

Western blots for hFR and caveolin with probes of anti hFR globulin and anti caveolin globulin were carried out as previously described (Kim et al., 2002). CHO cells (2×10^6 cells/35 mm well) plated overnight were washed twice with 2 ml of PBS and scraped into 1 ml PBS (pH 7.4). The cells were pelleted and solubilized with PBS and 1% TX-100. The samples were spun in a microfuge. The supernatant was assayed for protein and equal amounts of protein (25 μg) were acetone precipitated. Acetone precipitations were pelleted and put in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (50 μg of 0.125 M Tris, pH 6.8, containing 2% SDS, 20% glycerol, 0.08% bromophenol blue and 100 mM DL-dithiothreitol). Samples were electrophoresed on a 12.5% SDS-PAGE, and transferred to nitrocellulose membrane. Western blots for hFR were incubated with mouse antibody to hFR (Transduction Lab.), and incubated with goat anti-rabbit IgG (Bio-Rad) conjugated with HRP, and analyzed by the ECL system (Amersham). Western blots for caveolin were incubated with mouse antibody to caveolin (Transduction Lab.), and incubated with HRP sheep anti-mouse IgG (Amersham), and analyzed by the ECL system (Amersham).

6. Folic acid binding assay

The 75% confluent of CHO cells in 35 mm polycarbonate transwells were washed twice with 3 ml of 10 mM Na-acetate buffer (pH 4.5) containing 150 mM NaCl to dissociate preoccupied folates on the cell surface hFR. The cells were then returned to neutral pH by washing with ice cold PBS (pH 7.4). For assaying cell surface binding activity of folic acid, cells were incubated in 2 ml of 9/10DMEM with ^{125}I-labeled histamine derivative of folic acid (1×10^5 cpm) on the apical or basolateral compartment of the filter chamber in an ice-cold water bath. After incubation, the filters were washed with cold PBS, excised and counted their cpm as previously described (Kim et al., 2004). We observed no detectable leakage of [^{125}I]-folic acid to the opposite side during the experiment.

7. PI-PLC sensitivity

For measuring of PI-PLC (Boehringer-Mannheim) releasability, CHO clone cells were incubated in plastic 35 mm tissue culture wells in 9/10 DMEM containing 10% FCS, PSF, and 250 μg/ml G418. Cell monolayers were rinsed once with 3 ml ice cold acid saline to remove surface bound folates, and neutralized by rinsing once with 3 ml ice cold PBS. Cells were incubated in media with or without PI-PLC (100 mU/1 ml/well) for 60 min at 37℃. To measure cell surface hFR levels in PI-PLC treated and untreated cells, cell surface folic acid binding assays were carried out as described above.

8. [^{3}H]5-MTHF internalization assay

CHO cells were grown in 35 mm polycarbonate transwells for 1 week in deplete minimal essential media without added folic acid (DMEM) containing 10% (v/v) FCS, PSF, and 250 μg/ml G418, resulting in a final folic acid concentration of approximately 1~10 nM. Internalization assays were carried out using [^{3}H]5-methyltetrahydrofolate ([^{3}H]5-MTHF) as previously described (Elwood et al., 1986; Kim et al., 2004). For the experiments, The filter grown CHO cell monolayers were washed with acid saline and PBS, and incubated with 500 nM [^{3}H]5-MTHF on the upper (apical) or the lower (basolateral) compartment of the filter chamber for 5 min at 4℃ and rinsed with cold PBS. The cells were then warmed to 37℃ for various times. After washing three times with PBS, the radioactivity on filters were counted. as previously described (Elwood et al., 1986; Kim et al., 2004).

RESULTS

1. Selection of stable CHO clones

We transfected pRc/CMV contained hFR cDNA (pRc/FR), caveolin cDNA (pRc/FR), or both pRc/FR and pRc/FR into CHO cells. We established stable transfectants of CHO cells that expressed high levels of hFR (A clones), caveolin (B clones), or both of hFR and caveolin (AB clones) proteins when compared to control CHO cells, which do

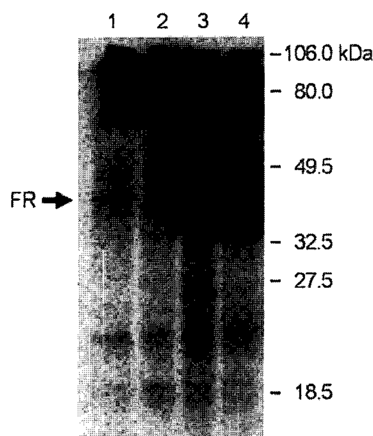

Fig. 1. Western blot analysis for expression of hFR. Western blots containing 25 μg protein from transfected FRT cells were detected using rabbit anti-hFR and HRP-goat anti-rabbit antibodies, and Amersham's ECL system, as described in 'Materials and Methods'. Wild type CHO cells (lane 1), CHO cells transfected with caveolin (lane 2), CHO cells transfected with hFR (lane 3), and CHO cells transfected with hFR and caveolin (lane 4).

not contain detectable levels of hFR and/or caveolin. as determined by Western blot analysis. Fig. 1 shows Western blots analysis of total cellular proteins probed with antibody to hFR. Wild type CHO cells do not express detectable levels of hFR protein (lane 1). Clone A cells (lane 2) and clone AB cells (lane 3) express high levels of hFR protein. In Western blots of CHO clones with caveolin antibody (Fig. 2), Clone B cells (lane 2) and clone AB cells (lane 3) express high levels of caveolin protein, but wild type CHO cells do not express detectable levels of caveolin protein (lane 1). Thus, we established three different phenotypes of stably transfected clones of clone A expressing only hFR protein, clone B expressing only caveolin protein and clone AB expressing both hFR and caveolin proteins. These three clones were used to characterize the expression of hFR, folic acid binding activity, transportation of folate and the potential interaction between hFR and caveolin.

2. Cell surface folic acid binding activity.

To determine the both apical and basolateral cell surface folic acid binding capacity of the folate receptor expressed in each CHO clone cells, the detergent-solubilized extracts were prepared from the cells and subjected to the binding assay using a ^{125}I-labeled histamine derivative of folic acid as described under "Materials and Methods". Fig. 3 shows

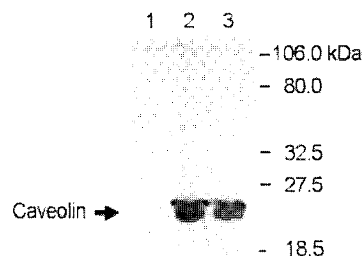

Fig. 2. Western blot analysis for expression of caveolin. Western blots containing 25 μg protein from transfected CHO cells were detected using mouse anti-caveolin and HRP-sheep anti-mouse antibodies, and Amersham's ECL system, as described in 'Materials and Methods'. CHO cells (lane 1), CHO cells transfected with caveolin (lane 2), CHO cells transfected with hFR and caveolin (lane 3).

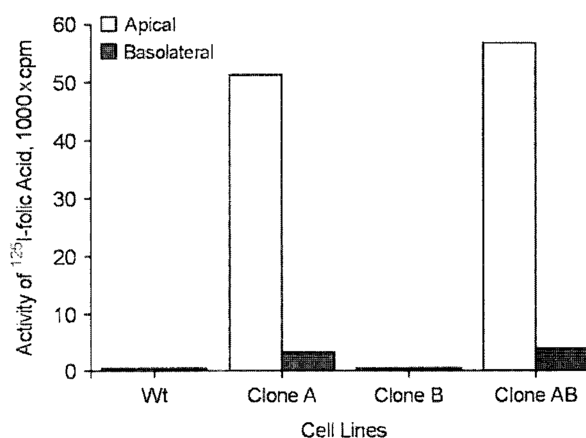

Fig. 3. Cell surface folic acid binding assays of 10 cm confluent plates of CHO cells (Wt) stably transfected with hFR (Clone A), caveolin (Clone B) and caveolin and hFR (Clone AB). Folic acid binding assays on detergent solubilized protein extracts were carried out as previously described by Kim et al (2004). The folic acid binding activity is expressed as cpm per μg protein.

that clones A and AB bind high levels of radiolabeled folic acid at their apical cell surface, but they bind much low levels of folic acid binding at their basolateral cell surface (6.8% in clone A and 7.6% in clone AB) compare to the binding at their apical surface (Clone A, Clone AB). The parental CHO cells (Wt) and clone B do not contain detectable levels of cell surface folic acid binding. Since the levels of folic acid binding at both sides are comparable between clones A and AB, these results show that hFR is expressed at the cell surface and binds folic acid independently of caveolin.

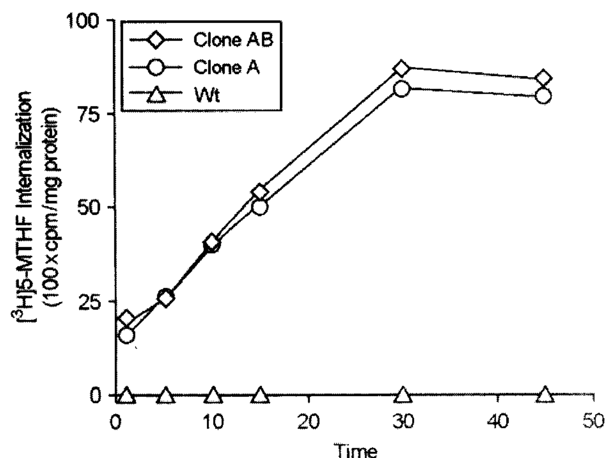

Fig. 4. [^{3}H]5-MTHF internalization in control CHO cells (Wt), CHO cells stably transfected with hFR (clone A) and transfected with caviolin and hFR (clone AB). [3H]5-MTHF internalization assays were carried as described in Materials and Methods. Cells were incubated in DMEM containing 10% FCS, PSF, and 250 μg/ml G418 with [^{3}H]5-MTHF on the lower (basolateral) compartment of the filter chamber for the various times at 37℃.

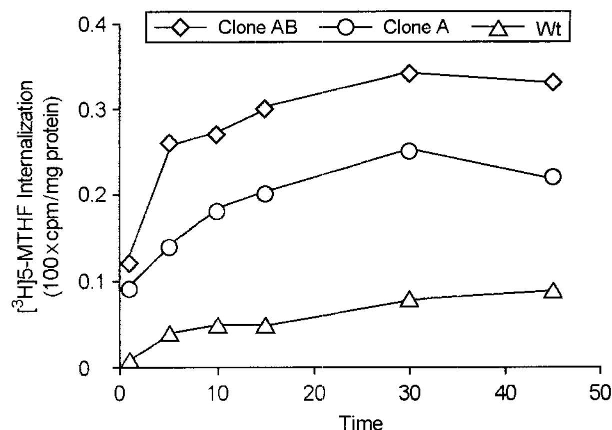

Fig. 5. [^{3}H]5-MTHF internalization in control CHO cells (Wt), CHO cells stably transfected with hFR (clone A) and transfected with caviolin and hFR (clone AB). [^{3}H]5-MTHF internalization assays were carried as described in Materials and Methods. Cells were incubated in DMEM containing 10% FCS, PSF, and 250 μg/ml G418 with [^{3}H]5-MTHF on the lower (basolateral) compartment of the filter chamber for the various times at 37℃.

3. PI-PLC releasability of folate receptor protein

To confirm that folate receptor protein in CHO transfectants is expressed in the same way as GPI-linked proteins on the both side of apical and basolateral as previously described (Kim et al., 2002; Kim et al., 2004), we carried out treatment of PI-PLC on the hFR expressed on the cell surface of clones A and AB. We measured that more than 96% of hFR on the apical cell surface in clone A and 85% in clone AB are released by PI-PLC. Wild type CHO cells and clone B cells do not contain detectable levels of hFR for measuring the PI-PLC sensitivity.

4. Internalization of ^{3}H-5MTHF

We measured the specific internalization of the circulating form of folate (^{3}H-5MTHF) to compare that the function of hFR at the both apical (Fig. 3) and basolateral (Fig. 4) cell surface in the transfected CHO cells. Clones A and AB that expressed high levels of hFR showed increased ^{3}H-5MTHF uptake at the apical side relative to wild type CHO cells (Fig. 3). The uptake of ^{3}H-5MTHF at the basolateral showed much lower (3.1% in clone A and 3.8% in clone AB) relative to apical (Fig. 4). Wild type CHO cells and clone B expressed caveolin only do not transport detectable amounts of folates at both sides (data not show). Since the kinetics of internalization of folic acid are comparable between clones A and AB, these results demonstrate that hFR can function independently of caveolin in CHO clone cells.

DISCUSSION

We carried out experiments to explore the expression and function of hFR and the effect of caveolin on the expression and function of hFR in CHO clones expressed high levels of hFR and/or caveolin protein. Also, we compared the ability of cell surface folic acid binding and internalization at apical and basolateral surface to each CHO clone cells. We observed that the wild type CHO cells do not express detectable levels of hFR and caveolin. In CHO cells transfected with hFR cDNA and/or caveolin cDNA, we observed that recombinant hFR and caveoin are expressed each their protein (Fig. 1 and 2) in CHO cells. The expression of hFR and caveolin in each CHO transfectants are identical in expression of them in other types of cells stably transfected with hFR and or caveolin (Matsue et al., 1992; Chung et al., 1993; Kim et al., 2002).

We conclude that the synthesis and sorting of hFR is independent of caveolin expression in CHO cells. We predicted that hFR would be expressed in the same way as other GPI-linked proteins on the apical surface (Kim et al.,

2004). We found that less than 93~92% of the hFR appear on the apical membrane and 7~8% of the hFR on the basolateral side of clone A and clone AB analyzed by cell surface [^{125}I]-folic acid binding assays (Fig. 3). The sensitivity of PI-PLC on hFR that we observed is more than 96% (in clone A) and 85% (in clone AB) of the hFR expressed on the apical cell surface in clones is released by PI-PLC. We and others reported that less than 30% of the transfected hFR is released with PI-PLC from either the apical or the basolateral sides in MDCK transfectants (Kim et al., 2002) and less than 50% of the hFR in KB cells is released with PI-PLC (Chung et al., 1995). Some researchers have suggested that the way of expression, the formation of the folate binding domain and structure of the hFR may be related to glycosylation of hFR in different types of cells (Conzelmann et al., 1988; Mayor et al., 2004). In some previous reports, the overexpression of caveolin lead to a decrease in the expression of GPI-anchored proteins (Mayor and Maxfield, 1995; Galbiati et al., 1998; Laurence et al., 2001; Sprenger et al., 2006). We do not yet know why the sensitivity of PI-PLC on the hFR expressed in other cell lines is different. Our data show that the releasability of hFR by PI-PLC in clone AB transfected with both hFR and caveolin is 11% lower than it in clone A only transfected with hFR. This result suggests that the expression of high levels of caveolin does not be involved in expression of hFR, but plays a role as a factor in PI-PLC releasing kinetics in CHO clone cells transfected with both hFR and caveolin.

We also demonstrated the specific internalization of the circulating form of folate (5-MTHF). The function of caveolin and the role of caveolae consisting of caveolin are unknown. Some researchers reported that the hFR and other GPI proteins have been found to cluster in caveolae consisting of caveolin (Mayor et al., 1994; Wu et al., 1997). The authors suggest that internalization of the folate receptor is by endocytosis mediated by clathrin-dependent and independent pathways. However, GPI-anchoring is important for intracellular signaling for several different proteins, and in most cases, cross-linking of the protein is a prerequisite for signaling (Miotti et al., 2000). Although the physiological activators of clustering hFR are not known, cross-linking may be involved in hFR function. Our data show that CHO clones stably transfected with hFR and/or caveolin show high levels of specific ^{3}H-5MTHF uptake from the apical surface during 30 min (Fig. 3), but the uptake of ^{3}H-5MTHF from the basolateral shows 3.1% in clone A and 3.8% in clone AB relative to apical (Fig. 4). These results suggest that It is possible to transport the folate at the basolateral surface of the cells overexpressed the both hFR and caveolin, and that the overexpression of caveolin cause on the ability of internalization of hFR.

In summary, We determined the cell surface folic acid binding activity on the both apical and basolateral surfaces in CHO clone cells. We also determined whether caveolin expression is a determinant of hFR function. Our present results suggest a possibility that the overexpression of caveolin can be a factor as a regulation of formation, processing and function.

Acknowledgements

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