Identification of dynein light chain road block-1 as a novel interaction partner with the human reduced folate carrier

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Ashokkumar B, Nabokina SM, Ma TY, Said HM. Identification of dynein light chain road block-1 as a novel interaction partner with the human reduced folate carrier. Am J Physiol Gastrointest Liver Physiol 297: G480–G487, 2009. First published July 1, 2009; doi:10.1152/ajpgi.00154.2009.—The reduced folate carrier (RFC) is a major folate transport system in mammalian cells. RFC is highly expressed in the intestine and believed to play a role in folate absorption. Studies from our laboratory and others have characterized different aspects of the intestinal folate absorption process, but little is known about possible existence of accessory protein(s) that interacts with RFC and influences its physiology and/or cell biology. We investigated this issue by employing a bacterial two-hybrid system to screen a BacterioMatch II human intestinal cDNA library using the large intracellular loop between transmembrane domains 6 and 7 of the human RFC (hRFC) as bait. Our screening has resulted in the identification of dynein light chain roadblock-1 (DYNLRB1) as an interacting partner with hRFC. Existence of a direct protein–protein interaction between hRFC and DYNLRB1 was confirmed by in vitro pull-down assay and in vivo mammalian two-hybrid luciferase assay and coimmunoprecipitation analysis. Furthermore, confocal imaging of live human intestinal epithelial HuTu-80 cells demonstrated co-localization of DYNLRB1 with hRFC. Coexpression of DYNLRB1 with hRFC led to a significant ($P < 0.05$) increase in folate uptake. On the other hand, inhibiting the endogenous DYNLRB1 with gene-specific small interfering RNA or pharmacologically with a specific inhibitor (vanadate) led to a significant ($P < 0.05$) decrease in folate uptake. This study demonstrates for the first time the identification of DYNLRB1 as a novel interaction partner with RFC and influences its physiology and/or cell biology of hRFC.

FOLATE IS AN IMPORTANT B-class vitamin, which plays a fundamental role in one-carbon metabolism and is essential for the synthesis of precursors of nucleic acids (15, 19). Folates are also essential for the conversion of methionine to S-adenosylmethionine, which is required for methylation reactions (4, 39). DNA methylation is an important epigenetic determinant of gene expression (3). Hence, cellular deficiency of this essential micronutrient leads to a disturbance in the normal physiology of the cell that ultimately manifests itself in the form of undesirable clinical abnormalities including neural tube defects (13), megaloblastic anemia (46), cardiovascular abnormalities (5), and cancer (10). In contrast to the adverse effects of folate deficiency, optimization of folate body homeostasis is effective in the prevention of neural tube defects (12, 45) and may also be beneficial in reducing the incidence of cardiovascular diseases (5), Alzheimer disease (38), and colorectal cancer (17).

Unlike bacteria and plants, mammals lack the cellular machinery to synthesize folate (6) and thus must obtain the vitamin from exogenous sources. Three different folate transport systems have been identified in mammalian cells: the folate receptors (1), the reduced folate carrier (RFC) (26, 37), and the proton-coupled folate transporter (PCFT) (31). The normal intestine expresses RFC and PCFT only (31, 33, 44). RFC is widely expressed in a variety of epithelial and nonepithelial cells (24, 42). The RFC protein is predicted to have 12 transmembrane domains with both the amino and carboxy termini predicted to be oriented intracellularly (7, 14). The RFC protein is also predicted to contain a long intracellular loop between transmembrane domains 6 and 7, which is believed to play a role in function and expression of the carrier protein at the cell membrane (23, 32, 36). Furthermore, the backbone of the RFC polypeptide has been shown to be important for membrane targeting and expression of the protein (25). Moreover, intracellular trafficking of the RFC polypeptide appears to involve distinct trafficking vesicles whose movement is critically dependent on intact microtubules (25).

Recent studies with other membrane transporters have identified an array of interacting proteins with these membrane proteins. These interacting proteins were shown to influence different aspects of the physiology and cell biology of these transporters (11, 16, 40). Little, however, is known about possible existence of interacting proteins with intestinal folate transporters (i.e., RFC and PCFT). Availability of such knowledge is important for better understanding of the normal intestinal folate uptake process. Also, a defect in the function of such an interactors may impact the overall vitamin absorption process. Thus in this study we focused on identifying possible interactors with hRFC using a bacterial two-hybrid system to screen a BacterioMatch II human intestinal cDNA library. Our screening effort has led to the identification of dynein light chain roadblock-1 (DYNLRB1) as an interacting partner with hRFC. We have confirmed this existence of such a direct interaction between DYNLRB1 and hRFC both in vitro (GST pull-down assay) and in vivo (luciferase assay, coimmunoprecipitation) and also showed that the interaction is important for the function and cell biology of hRFC.

MATERIALS AND METHODS

All chemicals, reagents, and kits were of analytical or molecular biology grade and were obtained from commercial sources. [3H]folic acid (specific activity: 20 Ci/mmol; radiochemical purity: 98%) was obtained from Moravek Biochemicals (Brea, CA). Anti-DYNLRB1 (anti-km23-1) polyclonal antibodies were kindly provided by Dr. [Address for reprint requests and other correspondence: H. M. Said, VA Medical Center-151, Long Beach, CA 90822 (e-mail: hmsaid@ucl.edu).]
DYNLRB1 INTERACTS WITH hRFC

K. M. Mulder (Pennsylvania State University College of Medicine, Hershey, PA). The human-derived intestinal epithelial Caco-2 and HuTu-80 cells and human-derived cervical epithelial HeLa-S3 cells were purchased from American Type Culture Collection (Manassas, VA) and were grown in DMEM or MEM supplemented with 10% FBS. HeLa R5 cell line is a RFC-null mutant and was a generous gift from Dr. I. D. Goldman (Albert Einstein Cancer Research Center, Bronx, New York).

_BacterioMatch II human intestinal cDNA library screening._ To search for proteins that may interact with the hRFC, the BacterioMatch two-hybrid system (Stratagene) was used to screen a _BacterioMatch II_ human intestinal cDNA library (Stratagene). A fragment of the hRFC encoding the large intracellular loop between transmembrane domains 6 and 7 (amino acids 204 to 264) was used as bait for screening and was cloned in frame into the _SalI/XhoI_ sites of the pBT vector to generate pBT-hRFC. cDNA library in the _pTRG_ plasmid was screened with pBT-hRFC as specified by the manufacturer. Briefly, the screening reporter competent cells were cotransformed with pBT-hRFC and _pTRG_-cDNA library plasmids and transformants were selected by their ability to grow on selective medium containing 5 mM of 3-amino-1,2,4-triazole (3-AT) and streptomycin. To validate the detected protein-protein interaction, competent cells were retransformed by use of _pTRG_ purified from the positive clones and recombinant pBT-hRFC.

_Mammalian two-hybrid assay._ To establish protein-protein interactions in vivo in mammalian cells, CheckMate Mammalian Two-hybrid System (Promega, Madison, WI) was used following manufacturer’s instructions. A fragment of the hRFC encoding the large intracellular loop between transmembrane domains 6 and 7 (amino acids 204 to 264) was cloned in frame in the _BamHI/XhoI_ sites of the pBIND fusion vector to generate Gal4 DNA binding domain fused to hRFC. The full coding sequence of the human DYNLRB1 was cloned in frame in the _BamHI/XhoI_ sites of pACT vector to produce the activation domain of herpes simplex virus type 1 VP16 protein fused separated by SDS-PAGE (8%), stained with Coomassie brilliant blue, tagged hRFC. Caco-2 cells (90% confluence) were transiently transfected with pFLAG-CMV-2-hRFC by use of Lipofectamine 2000 (Invitrogen). Cells were lysed after 48–72 h post transfection in a buffer containing 50 mM Tris·HCl, pH 7.4, containing 100 mM KCl, 1% Triton X-100 (lysis buffer), and protease inhibitors (2 mM phenylmethlysulfonyl fluoride, 1 µg/ml aprotinin, and 2.5 µg/ml leupeptin). Postnuclear extracts (0.1–0.5 mg of protein) from transfected and nontransfected (negative control) cells were incubated with 100 µl anti-FLAG M2 affinity gel (Sigma) suspension overnight at 4°C on rotating wheel. Gels were washed three times with lysis buffer and another three times with lysis buffer without Triton X-100. Final pellet was resuspended in SDS-PAGE sample buffer and analyzed by Western blotting (2) using the anti-FLAG M2 monoclonal antibody (Sigma) at 1:5,000 dilutions and anti-DYNLRB1 (anti-km23-1) polyclonal antibody at 1:1,000 dilutions.

_Silencing of DYNLRB1 with siRNA._ Caco-2 cells were transiently transfected with DYNLRB1 gene-specific small interfering RNA (siRNA) or scrambled siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) using Lipofectamine 2000 (Invitrogen). To check the silencing effect of siRNA on DYNLRB1 mRNA, semi-quantitative RT-PCR was performed with total RNA isolated by using the TRIzol reagent (Invitrogen) and RT reactions from appropriate cell samples after 48 h of siRNA transfection. Briefly, 5 µg of total RNA was reverse transcribed with oligo(dT) primers by using Superscript II (Invitrogen). Samples were then diluted with sterile water, and two different dilutions were used for each PCR with specific primers for hRFC, DYNLRB1, and the housekeeping gene 18S rRNA. For hRFC, the primers were forward, 5′-ACCCGACTACCTGGCGCTACA-3′; reverse, 5′-GCCATGTT-GACGTCTGTAGAA-3′. For DYNLRB1, the primers were forward, 5′-GGGAGGTAGTGAC-GAAAATAACAAAT-3′; reverse, 5′-TTCGCCCTCAATGGATC-GTC-3′. Conditions for semi-quantitative RT-PCR were 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min (33 cycles). The products were analyzed on 2% agarose gels, the images were captured by use of an Eagle Eye II system, and the amplified RT-PCR products were normalized to amplified 18S rRNA controls as described previously (2).

_Folic acid uptake._ Uptake of folic acid by confluent monolayers was performed as described by us previously (2) with some modifications. Briefly, cells were incubated at 37°C for 5 min in Krebs-Ringer (K-R) buffer (in mM: 133 NaCl, 4.93 KCl, 1.23 MgSO₄, 0.85 CaCl₂, 5 glucose, 5 glutamine, 10 HEPES, pH 7.4) in the presence of labeled and unlabeled folic acid (final concentration of 2 µM). Reaction was terminated by the addition of 2 ml of ice-cold K-R buffer followed by immediate aspiration. Cells were then rinsed twice with ice-cold buffer, lysed with 1 ml of 1 N NaOH, neutralized with 10 N HCl, and then counted for radioactivity. Protein content of cell digests was determined by using a Bio-Rad kit (Bio-Rad, Richmond, VA).

_Confocal imaging._ Colocalization of hRFC with DYNLRB1 was performed by live cell confocal imaging following transient transfection of the human intestinal epithelial HuTu-80 cells with hRFC-GFP and DsRed-DYNLRB1 plasmids. Monolayers were grown on glass-bottomed petri dishes (MatTek, Ashland, MA), and imaging was performed with a Nikon C-1 confocal scanner head attached to Nikon inverted phase contrast microscope. Fluorophores were excited by a 488-nm line from an argon ion laser, and a 543-nm line from a HeNe ion laser-emitted fluorescence was monitored with a 515±30-nm band-pass (GFP) or at 570±50-nm long-pass (DsRed) filter.

_Biotinylation of cell surface proteins._ Biotinylation of cell surface proteins was performed essentially as described by Zimnicka et al. (49) with minor modifications. Briefly, HuTu-80 monolayers were exposed from surface with Sulfo-NHS-SS-biotin (no. 21331, Pierce Biotechnology, Rockford, IL) diluted into a biotinylation buffer (PBS, pH 7.5, supplemented with 10 mM triethanolamine, 2 mM CaCl₂, and 150 mM NaCl) to the working concentration of 1 mg/ml, then incubated for 1 h at 4°C in horizontal motion. Cells were then stained with 0.5% biotinylated streptavidin (Pierce) followed by 10 min incubation with 0.1% Triton X-100 (lysis buffer), and protease inhibitors (2 mM phenylmethlysulfonyl fluoride, 1 µg/ml aprotinin, and 2.5 µg/ml leupeptin). Postnuclear extracts (0.1–0.5 mg of protein) from transfected and nontransfected (negative control) cells were incubated with 100 µl anti-FLAG M2 affinity gel (Sigma) suspension overnight at 4°C on rotating wheel. Gels were washed three times with lysis buffer and another three times with lysis buffer without Triton X-100. Final pellet was resuspended in SDS-PAGE sample buffer and analyzed by Western blotting (2) using the anti-FLAG M2 monoclonal antibody (Sigma) at 1:5,000 dilutions and anti-DYNLRB1 (anti-km23-1) polyclonal antibody at 1:1,000 dilutions.
quenched with PBS containing CaCl2, MgCl2, and 100 mM glycine for 20 min at 4°C. Cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris·HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100). After centrifugation, the supernatant was incubated overnight in streptavidin agarose and then washed three times with lysis buffer. The streptavidin agarose beads were spun down, and sample loading buffer for SDS-PAGE was added. Separated proteins were probed with anti-hRFC antibodies and visualized with enhanced chemiluminescence reagent.

Statistical analysis. Experimental points of transport studies are means ± SE of multiple separate uptake determinations and were expressed in terms of either femtomoles or picomoles per milligram protein per 5 min. Carrier-mediated folate uptake was determined as described by us previously (2). Statistical analysis was performed by the Student’s t-test or one-way ANOVA with statistical significance being set at 0.05. All transfection studies, semiquantitative PCR, and Western blot analysis were performed on at least three separate occasions with comparable results. Data presented are from a representative set of experiments.

RESULTS

Identification of DYNLRB1 as a potential interacting protein partner of hRFC using bacterial two-hybrid assay. We used the bacterial two-hybrid system to screen a BacterioMatch II human intestinal cDNA library to search for proteins that may interact with the hRFC. The large intracellular loop between transmembranes 6 and 7 (amino acids 204 to 264) of hRFC was used as the bait in screening since it has been reported to play a critical role in function and expression of the RFC at the plasma membrane (23, 32, 36). The BacterioMatch II human intestinal cDNA library (primary size 1.67 × 106; Stratagene) was screened with the generated recombinant pBT-hRFC plasmid. We screened 2.1 × 106 clones and identified 55 positive clones grown on the selective screening medium containing 5 mM of 3-AT. These positive clones were then subjected to further screening on a dual selective screening medium (5 mM 3-AT and streptomycin), and 18 positive clones were identified. To validate the detected protein-protein interactions, we performed retransformation of the reporter strain using pTRG purified from positive clones and recombinant pBT-hRFC. Our results showed the identification of eight clones that reproducibly grow on selective screening medium (5 mM 3-AT) when cotransformed with the bait protein and that failed to grow on selective screening medium when cotransformed with the empty pBT vector. Among the eight identified clones, one clone was found to have a full sequence that is identical to the open reading frame of the dynein road block light chain 1

Fig. 1. Protein-protein interaction between human reduced folate carrier (hRFC) and dynein light chain road block-1 (DYNLRB1). Each pair of plasmids (A, pBT-LGF2 + pTRG-Gal 11p; B, pTRG-DYNLRB1 + pBT-hRFC; C, pTRG-DYNLRB1 + pBT-Vector) was cotransformed into BacterioMatch II validation reporter-competent cells. Bacterial transformants were grown on the selective screening medium [5 mM 3-amino-1,2,4-triazole (3-AT)]. The known interaction between LGF2 and Gal 11p (A) serves as a positive control.

Fig. 2. Interaction of hRFC and DYNLRB1 in vivo: mammalian 2-hybrid luciferase assay. Plasmids were transfected along with the pG5luc vector into HeLa S3 cells. Cells were lysed after 48 h of transfection, and Renilla-normalized Firefly luciferase activity was determined by using the dual luciferase assay system. Data are presented as means ± SE of at least 3 independent experiments and Firefly luciferase expression given in folds over the background (set arbitrarily at 1). *P < 0.01.

Fig. 3. Pull-down assay of hRFC from human intestinal epithelial Caco-2 cells by GST-DYNLRB1. A: generation and purification of GST-DYNLRB1 fusion protein and GST. Extracts from BL-21 Escherichia coli cells harboring recombinant pGEX-4T-1 (1), purified GST (2), pGEX-4T-1-DYNLRB1 (3), purified GST-DYNLRB1 (4), and marker (M). Protein samples were run on SDS-PAGE and stained with Cooamassie blue. B: Caco-2 cells lysate was incubated with glutathione-Sepharose 4B beads adsorbed with GST-DYNLRB1 or GST alone (negative control). Proteins bound to the beads were separated, washed and analyzed by Western Blotting using anti-hRFC antibodies. 1, Caco-2 cell lysate; 2, proteins bound to GST-DYNLRB1; 3, proteins bound to unfused GST.
hRFC and DYNLRB1 interact in vivo in mammalian two-hybrid luciferase assay. We examined the interaction of hRFC with DYNLRB1 in mammalian cells by using a mammalian two-hybrid system. In this system the interaction between the two test proteins expressed as GAL4 and VP16 fusion constructs results in transcription of the Firefly luciferase gene. Fragment of the hRFC encoding the large intracellular loop between transmembrane domains 6 and 7 (amino acids 204 to 264) was cloned in frame into the pBIND fusion vector to generate a fusion complex with Gal4 DNA binding domain. The full coding sequence of the DYNLRB1 was cloned in frame into the pACT vector to produce the activation domain of herpes simplex virus type 1 VP16 protein fused to DYNLRB1. HeLa S3 cells were cotransfected with pBIND-hRFC and pACT-DYNLRB1 plasmids along with the pG5luc vector, and 48 h posttransfection Renilla-normalized Firefly luciferase activity was determined. Our results (Fig. 2) showed the significant increase (~6-fold) in luciferase activity of cells cotransfected with hRFC and DYNLRB1 fusion constructs compared with negative controls. Thus DYNLRB1 appears to interact with the hRFC in mammalian cells, which confirms our previous findings in bacterial cells with a bacterial two-hybrid system.

GST-DYNLRB1 fusion protein binds with hRFC in human intestinal epithelial cells (GST pull-down assay). To further confirm the existence of the interaction between hRFC and DYNLRB1 in human intestinal cells, we performed in vitro GST pull-down assay using a GST-fused DYNLRB1 and lysate from the Caco-2 cells. For this, we generated and affinity purified GST-DYNLRB1 fusion protein and GST from BL-21 E. coli cells harboring recombinant pGEX-4T-1 and pGEX-4T-1, respectively (Fig. 3A). Caco-2 cells lysate was incubated...
with glutathione-Sepharose 4B beads adsorbed with GST-DYNLRB1 or GST alone (negative control). The bound proteins were eluted with glutathione and processed for Western blotting using anti-hRFC antibodies previously generated and characterized in our laboratory (2). The results (Fig. 3B) showed that GST-DYNLRB1 pulled down the hRFC from Caco-2 extract, whereas GST did not bind with the hRFC. These data suggest that DYNLRB1 indeed binds with hRFC in intestinal Caco-2 cells extracts.

Interaction between DYNLRB1 and hRFC in vivo in human intestinal epithelial cells (coimmunoprecipitation assay). We also studied whether the interaction between DYNLRB1 and hRFC occurred in vivo in Caco-2 cells by coimmunoprecipitation. To this end, we cloned the open reading frame of hRFC into pFLAG-CMV-2 expression vector to generate FLAG-tagged hRFC. Caco-2 cells were transiently transfected with pFLAG-CMV-2-hRFC, and after 48–72 h posttransfection cell extracts were used for coimmunoprecipitation assay. Anti-FLAG M2 affinity gel was incubated with extracts prepared from transfected and nontransfected (negative control) Caco-2 cells, and then the bound proteins (FLAG-hRFC immunoprecipitates) were analyzed by Western blotting using the anti-FLAG M2 monoclonal antibody and anti-DYNLRB1 polyclonal antibodies. The results (Fig. 4) showed that the FLAG-hRFC immunoprecipitate obtained from pFLAG-CMV-2-hRFC transfected cells contains DYNLRB1. In contrast, DYNLRB1 from nontransfected cells was not detected among the proteins bound to anti-FLAG M2 affinity gel. These data suggest that hRFC and DYNLRB1 interact in vivo in human intestinal epithelial Caco-2 cells.

Functional implications of the interaction between hRFC and DYNLRB1. On the basis of the identified physical interaction between DYNLRB1 and hRFC, we investigated the consequences of this interaction on the functionality of hRFC. This was performed by measuring the [3H]folic acid uptake (2H9262M; pH 7.4) in HeLa R5 cells transfected with hRFC in the presence and absence of DYNLRB1. The results showed that the coexpression of hRFC with DYNLRB1 leads to a significant (P < 0.05) increase in RFC-mediated folic acid uptake compared with cells transfected with hRFC alone (Fig. 5). Similarly, uptake of folic acid (2 H9262M; pH 7.4) in the human intestinal epithelial HuTu-80 cells was significantly (P < 0.05) increased with cotransfecting hRFC and DYNLRB1 compared

![Fig. 7](http://ajpgi.physiology.org/)

**A:** Effect of vanadate treatment on carrier-mediated folic acid uptake and on cell surface expression of hRFC. A: HuTu-80 cells cotransfected with hRFC-pFLAG and DYNLRB1-pFLAG were treated with (+) or without (−) vanadate (Van; 100 μM) for 6 h at 37°C. A: initial rate (5 min) of [3H]folic acid (2 μM; pH 7.4) uptake was measured after 24 h of vanadate pretreatment. Values are means ± SE of 3–4 separate uptake determinations. **P < 0.05. B:** transfected HuTu-80 cells grown in the presence or absence of vanadate were exposed to sulfo-NHS-SS-biotin for 1 h at 4°C. Cells were then quenched and lysed. Biotinylated proteins were extracted with sample loading buffer for SDS-PAGE and analyzed by Western blotting. The hRFC proteins were detected with anti-hRFC specific antibodies. The biotinyalted membranous hRFC levels were normalized to the total amount of cellular hRFC. C: densitometric analysis of cell surface expression of hRFC with or without vanadate treatment. Image and data shown are representative of 3 separate sets of experiments.

![B](http://ajpgi.physiology.org/)

(1) Membrane biotinylated

![C](http://ajpgi.physiology.org/)
with uptake by the cells transfected with hRFC alone (6.84 ± 0.6 and 5.2 ± 0.2 pmol/mg protein, respectively).

In another approach, we examined the effect of inhibiting the endogenous DYNLRB1 using molecular (gene silencing with use of gene-specific siRNA) and pharmacological (vandate treatment) approaches on functionality of the endogenous hRFC in intestinal epithelial cells. Results of the gene-knockdown approaches showed a significant \((P < 0.05)\) decrease in the uptake of folic acid (2 \(\mu\)M; pH 7.4) in Caco-2 cells treated with DYNLRB1 siRNA compared with those treated with scrambled siRNA as well as that of control (Fig. 6A). Effectiveness and specificity of the DYNLRB1 knockdown was verified by semiquantitative RT-PCR, which showed a marked reduction in mRNA levels of DYNLRB1 in the cells treated with DYNLRB1 gene-specific siRNA compared with control. On the other hand, no change in hRFC and 18S rRNA expression levels was observed upon DYNLRB1 siRNA treatment (Fig. 6B). In the pharmacological approach, we used vanadate (an agent that disrupts the function of dynein-related proteins; Ref. 28) to study the effect of inhibiting DYNLRB1 on folic acid uptake (2 \(\mu\)M; pH 7.4) in HuTu-80 cells cotransfected with hRFC and DYNLRB1. The results showed significant \((P < 0.05)\) reduction in folic acid uptake in vanadate-treated compared with untreated cells (Fig. 7A). We also examined the effect of DYNLRB1 inhibition on cell surface expression of hRFC in HuTu-80 cells. The results demonstrated that cell surface expression of hRFC to be significantly \((P < 0.05)\) decreased in vanadate-treated compared with untreated control cells (Fig. 7B). Total cellular levels of hRFC, however, were not affected by the vanadate treatment, indicating that vanadate did not affect the total hRFC expression level or processing. We also examined the effect of vanadate on the colocalization of DYNLRB1 with hRFC using live cell confocal imaging. As expected, DYNLRB1 colocalized with hRFC in control untreated HuTu-80 cells (Fig. 8A). However, in vanadate-treated cells, a marked decrease in DYNLRB1 colocalization with hRFC was clearly evident (Fig. 8B).

**DISCUSSION**

Understanding the cellular and molecular mechanisms involved in the intestinal folate absorption process and their regulation is of significant physiological and nutritional importance. This has been the subject of interest in our laboratory and others for over three decades (reviewed in Refs. 34 and 47). Whereas a significant progress has been made so far in our understanding of the folate uptake systems involved (RFC and PCFT), very little is currently known about possible existence of accessory proteins that may influence the physiology and cell biology of the intestinal folate uptake systems. In this study, we focused on hRFC and used bacterial two-hybrid analysis to screen human intestinal cDNA library. Our effort led to the identification of DYNLRB1 as an interacting partner with hRFC. The existence of direct interactions between hRFC and DYNLRB1 was confirmed in vitro by GST-pull-down assay as well as in vivo in mammalian cells by two-hybrid...
luciferase and coimmunoprecipitation assays. Furthermore, confocal imaging of living human intestinal epithelial cells expressing hRFC-GFP and DsRed-DYNLRB1 showed colocalization of the two proteins, thus providing further support for existence of interaction. Following establishment of existence of direct interaction between hRFC and DYNLRB1 in vitro and in vivo, we then moved to investigate the effect of such an interaction on physiology and cell biology of hRFC. Physiological (functional) consequences of the interaction between hRFC with DYNLRB1 was examined by coexpressing the two proteins in HeLa R5 cells (a cell line that does not express hRFC; Ref. 9) and in human intestinal epithelial HuTu-80 cells followed by examining the effect of this coexpression on hRFC transport function, i.e., on [3H]folic acid uptake. Uptake studies were performed by using a folic acid concentration of 2 μmol/l at pH 7.4 to eliminate the contributions of the folate receptor (which functions in the nanomolar range; Ref. 27) and PCFT (which functions at acidic pH; Ref. 48) to the uptake process. The results showed that coexpressing hRFC with DYNLRB1 leads to a significant increase in folic acid uptake by both HeLa R5 and HuTu-80 cells compared with cells expressing hRFC alone. These findings clearly suggest a role for DYNLRB1 in the function of hRFC. This was confirmed by the studies in which DYNLRB1 was inhibited by molecular (gene-specific siRNA) and pharmacological (vanadate treatment) means. Knocking down the endogenous DYNLRB1 by gene-specific siRNA (verified by PCR) led to a significant inhibition in folic acid uptake. Similarly, treatment with vanadate led to a significant inhibition in folic acid uptake by cells coexpressing hRFC and DYNLRB1. These findings further confirm a role for DYNLRB1 in hRFC transport physiology.

To understand the role of DYNLRB1 in hRFC cell biology, we examined the effect of inhibiting DYNLRB1 (with vanadate) on membrane expression of hRFC as well as on intracellular colocalization of the two proteins. Effect of DYNLRB1 inhibition on cell surface expression of hRFC in intestinal epithelial cells was investigated by performing membrane biotinylation assay before and after vanadate treatment. The results showed a marked decrease in the levels of hRFC membrane expression in vanadate-treated compared with untreated control cells. This decrease in membrane expression of hRFC may explain the significant decrease in folic acid uptake observed upon inhibition of DYNLRB1. In line with these findings is the observation that DYNLRB1 inhibition also leads to a marked decrease in its colocalization with hRFC. These observations imply a role for DYNLRB1 in the cell biology and membrane expression of hRFC, an effect that ultimately affects the physiology of hRFC. This role for DYNLRB1 in hRFC physiology resembles the role played by another member of the light chain dynein complex (Tctex-1) that interacts with voltage-dependent anion-selective channel 1 and influences its physiology and biology (35). It is interesting to mention here that the expression of DYNLRB1 is upregulated in hepatocellular carcinoma (18) and that overexpression of this protein promotes cell survival and inhibits apoptosis in differentiated PC12 cells (8).

In summary, our results demonstrate for the first time the identification of DYNLRB1 as an interacting protein partner with the hRFC in human intestinal epithelial cells and show that this interaction may influence both the cell biology and physiology of hRFC.

GRANTS

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