Pathol. Oncol. Res. (2009) 15:31–36
DOI 10.1007/s12253-008-9085-1

ORIGINAL PAPER

Oncogene Functions of FHL2 Are Independent from NF-κBIα in Gastrointestinal Cancer

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Received: 28 January 2008 / Accepted: 7 July 2008 / Published online: 28 August 2008

Abstract Four and a half of LIM-only protein 2 (FHL2) is an adaptor protein that can interact with many transcription factors and thus plays a variety of biological functions. Previous studies by our group have demonstrated that suppression of FHL2 was capable of inducing tumor cell differentiation, and inhibiting the growth of experimental gastric and colon cancers. Therefore, FHL2 appears to function as an oncogene. In order to further explore the mechanisms of how FHL2 is involved in tumorigenesis, we attempted to test whether FHL2 has any direct association with nuclear factor (NF-κB), the most important transcription factor involved in apoptosis, inflammation, and carcinogenesis. Using an Yeast Two Hybrid (Y2H) screening system, we have shown that FHL2 may have an interaction with NF-κBIα, the coding gene for IκBα which is the most potent endogenous inhibitor for NF-κB activation. However, subsequent studies using co-immunoprecipitation and co-localization failed to confirm the Y2H finding. Down-regulation of FHL2 by FHL2-siRNA down-regulated the expression of NF-κB p65. We therefore concluded that under the physiological condition, FHL2 may activate NF-κB pathway, even though such an activation may not be mediated by a direct binding of FHL2 to NF-κB inhibitor protein IκB.

Keywords FHL2 · NF-κB · NF-κBIα · Interaction · Gastric cancer · Colon cancer

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Introduction

Four and a half of LIM-only protein 2 (FHL2), also known as down-regulated in rhabdomyosarcoma LIM protein is the second member of a small family of five proteins with four and a half LIM domains. [1] FHL2 is expressed in many normal human tissues such as heart, ovary, kidney, prostate, testis, small intestine, and colon, [1–3] but its expression is often deregulated (either over-expressed or down-regulated) in many types of cancers. Compared to the non-malignant counterparts, FHL2 is often over-expressed in some cancers including prostate cancer, ovarian cancer, hepatoblastomas, gastric cancer, colon cancer, and breast cancer. [4–9] However, FHL2 was found to be down-regulated in malignant rhabdomyosarcoma. [2] Therefore, the expression and function of FHL2 may be cell type-specific.

FHL2 exerts its function as a transcriptional cofactor through interaction with a broad range of transcription factors by forming different protein complexes in different cell types. FHL2 has been found to interact with many proteins to modulate their transcriptions, including but not limited to androgen receptor, cyclic adenosine monophosphate response element-binding protein, integrins, β-catenin, presenilin 2, ERK2, AP-1, SKI, CBP/p300, BRCA1, Forkhead box class O 1, and Proline-, glutamic acid-, and leucine-rich protein-1 (PELP1). [7–19]

Ectopic expression of FHL2 potently triggered apoptosis in cell lines of different origin including COS1 cells, NIH 3T3 cells, and RD cells. [3] However, FHL2 failed to show any pro-apoptotic role in HeLa and HEK293 cells, possibly due to its stimulation of nuclear factor (NF)-κB. [20] In breast cancer, over-expression of FHL2 may contribute to cancer development by mediating transcriptional activation of MAPK target gene p21. [9] Our group has previously demonstrated that suppression of FHL2 induces cell

differentiation and inhibits tumorigenesis of gastrointestinal cancer cells. [6] These studies suggested that FHL2 may exert different biological function in different cell types. Indeed, FHL2 has a dual nature: it can function as repressor or activator of transcriptional activity depending on the cell type, and as a result, it may act as an oncoprotein or as a tumor suppressor. [21]

The different biological consequences of FHL2 may be related to its interaction with different partner proteins in different cell types. NF-κB is an important transcription factor regulating many cellular processes such as apoptosis, proliferation, and cellular transformation. NF-κB is also critically implicated in tumorigenesis by regulating expression of genes that are involved in invasion, angiogenesis and metastasis. We are therefore interested in elucidating whether FHL2 is involved in human carcinogenesis through interaction with NF-κB. Although FHL2 was reported to stimulate TRAF-induced expression of NF-κB-responsive promoters in HEK293 cells, FHL2 by itself was not sufficient to induce the NF-κB responsive promoter. [20, 22] The direct interaction between FHL2 and NF-κB has never been addressed to the best of our knowledge.

NF-κB is a homodimer or heterodimer composed of several subunits p50, p52, p65, RelB or c-Rel. In quiescent cells NF-κB is bound to the inhibitor of NF-κB (IκB) proteins, most notably IκBα, and this complex is kept inactive in the cytoplasm. Any stimuli that cause ubiquitination and degradation of IκBα would unmask the nuclear localization signal of NF-κB which is then free to translocate into the nucleus whereby it binds to the promoter of its target genes and initiate transcription. IκBα is a potent inhibitor for NF-κB and targeting NF-κB by modulating IκBα has become one of the approaches for cancer therapy. [23–25]

NF-κBIα (nuclear factor of kappa light chain gene enhancer in B cells inhibitor, alpha) is the coding gene for IκBα. Certain variant of NF-κBIα such as NF-κBIα with AG genotype may be associated with an increased risk of developing colorectal cancer. [26] How NF-κB interacts with other proteins to regulate gene expressions in carcinogenesis is an important aspect of cancer research.

In this brief study, we aimed to test whether FHL2 interacts with NF-κB regulator NF-κBIα.

Materials and Methods

Chemicals and Reagents

Anti-FHL2 antibody was purchased from MBL (Medical and Biological Laboratories CO., LTD.). Anti-IκBα (C-21) and anti-NF-κB p65 were from Santa Cruz (Santa Cruz, CA, USA). Anti-cMyc antibody (9E10) was purchased from Invitrogen (Invitrogen, CA, USA). All horse-radish-peroxidase-conjugated secondary antibodies and FITC conjugated anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa-Cruz, CA, USA). Anti GAPDH was purchased from Abcam (Cambridge, MA, USA).

Cell Lines and Culture Conditions

Human gastric and colon cancer cell lines AGS, DLD1, LOVO, and HCT116 cells, as well as HeLa cells were purchased from ATCC (Rockville, MD, USA). All cells were cultured in Dulbecco's modified Eagle's medium (GIBCO BRL, USA) supplemented with 10% heat inactivated fetal bovine serum (GIBCO BRL), 4 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37°C with an atmosphere of 5% CO_2.

Plasmids and Constructs

Vector plasmids of pEGFP-N1 and pCMV-Tag 3B were purchased from Clontech and Stratagene, respectively. pEGFP-N1-FHL2 was generated by cloning polymerase chain reaction (PCR)-generated full-length cDNA of FHL2 into the *Hin*dIII site of the pEGFP-N1 vector. pCMV-Tag 3B-IκBα was constructed by inserting the reverse transcript-PCR (RT-PCR)-generated full-length cDNA from DLD1 cells into the *Hin*dIII site of the pCMV-Tag 3B vector harboring a MYC epitope sequence. FHL2-siRNA and Luc-siRNA were designed and synthesized by Proligo (Singapore).

Transient Transfection

For transient transfection, 4 μg of pEGFP-N1-FHL2 or pCMV-Tag 3B-IκBα were mixed with 250 μl of serum- and antibiotic-free DMEM containing 10 μl LipofectAMINE2000 reagent and incubated at room temperature (RT) for 20 min. The mixtures were gently overlaid onto cell monolayers in six-well tissue culture plates pre-incubated under serum-free conditions for 20 min and incubated at 37°C culture condition. After 4 h, the DNA/liposome complex was removed, and the complete growth medium was replenished. Transfection of cells with FHL2-siRNA or Luc-siRNA was performed in the similar fashion except Oligofectamine was used instead of LipofectAMINE2000. Whole cell lysates were prepared 48 h later to assess the protein expression by Western blot.

Yeast two-hybrid screen

A standard yeast two-hybrid (Y2H) assay was performed as reported. [8, 23] Briefly, a bait plasmid was generated by inserting a PCR-amplified cDNA fragment encoding the full length FHL2 sequence into the *Nde*I–*Eco*RI restriction

sites of pAS2–1 (Clontech). The resultant plasmid pAS2-FHL2 and a human placental cDNA prey library cloned into the pACT2 vector (Clontech) were co-transformed into the yeast reporter strain PJ69a (Clontech), according to the manufacturer's instruction. Selection was made by growing the transformant on histidine-, adenine-, leucine-, tryptophan-free media and by the expression of the reporter gene LacZ, as evaluated in a β-galactosidase filter assay. cDNA clones from positive colonies were isolated, transferred into XL1 Blue bacteria and identified by cDNA sequencing.

Subcellular Localization of FHL2 and NF-κBIα

For detection of exogenous FHL2 and NF-κBIα, HeLa cells were cultured in six-well plates, co-transfected with pEGFP-N1-FHL2 and pCMV-Tag 3B-IκBα (at a ratio of 1:1) for 48 h. Cells were fixed with 4% phosphonoformic acid in phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100/PBS and blocked with 3% bovine serum albumin/PBS. Cells were then incubated with anti-NF-κBα at RT for 1 h, followed by incubation with a rhodamine-conjugated secondary antibody (Santa Cruz Biotechnology). For detection of endogenous FHL2 and NF-κBIα, cells were fixed with 3.7% paraformaldehyde, incubated with anti-FHL2 and anti-NF-κBIα, followed by incubation with fluorescein isothiocyanate-labeled secondary antibody (for FHL2) or rhodamine-conjugated secondary antibody (for NF-κBIα) at RT. Cells were washed and visualized using Zeiss Axioscop fluorescence microscope. Photographs were taken at the same field under the same magnification using different filters to obtain the green and red signals. Co-localization is indicated by the presence of both green and red color in the same cells.

Co-immunoprecipitation and Western Blot

For detection of endogenous FHL2/IκBα complex in DLD1 cells, cells were cultured in 6-well plates, washed twice with phosphate-buffered saline and lysed in 0.8 ml radioimmunoprecipitation assay buffer (Sigma) supplemented with 1:100 protease inhibitor cocktail and phenylmethanesulfonylfluoride (Sigma) and incubated on ice for 30 min. The lysate was centrifuged at 14,000 rpm for 30 min at 4°C, and the supernatant was used for subsequent immunoprecipitation experiments. For immunoprecipitation, 6 μg of monoclonal anti-FHL2 antibody or 2 μl of corresponding pre-immune serum diluted 1:10 was added to 0.8 ml of cell lysates and incubated for overnight at 4°C. Fifty microliter of pre-washed Protein G Sepharose beads (Roche) was then added to the each immunoprecipitate and mixed gently for 4 h at 4°C. The mixtures were centrifuged, eluted by 2× sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer, and resolved on SDS–polyacrylamide and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) for subsequent Western blot analysis by the standard procedures. Five microliter of the crude extract (input) was used for detecting protein expression levels. A reciprocal immunoprecipitation experiment was also performed using polyclonal anti-IκBα antibody to capture the FHL2/IκBα complex (if any) and the precipitates were detected by anti-FHL2 antibody. The membranes were then incubated with appropriate secondary antibodies, followed by detection with enhanced chemiluminescence assay (Amersham Pharmacia Biotech).

Results

Endogenous Expression of FHL2 and NF-κBIα

We first used Western blot to detect the endogenous expression of FHL2 and NF-κBIα in several gastric and colon cancer cell lines. Both FHL2 and NF-κBIα are expressed at abundant levels in the cell lines tested (Fig. 1a).

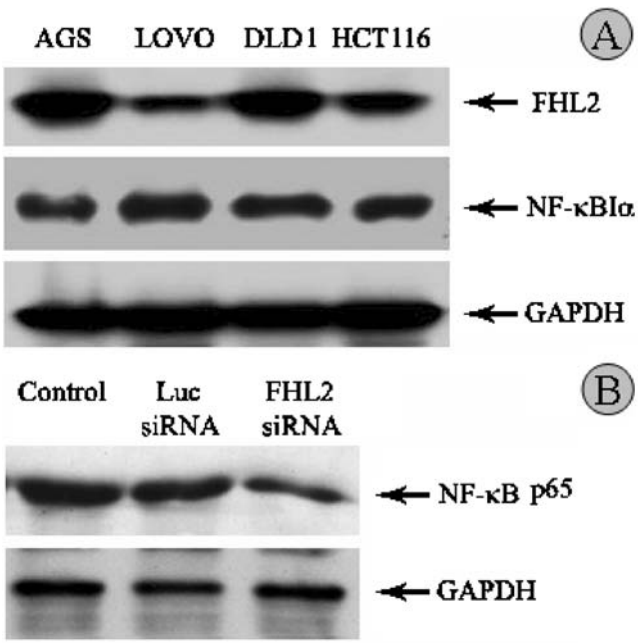

Fig. 1 **a** Expression of endogenous *FHL2* and *NF-κBIα* in gastric and colon cancer cell lines by Western blot. Gastric cancer cell line *AGS*, colon cancer cell lines *LOVO*, *DLD1*, and *HCT116* were cultured in RPMI1640, and cell lysates were prepared as described in the "Materials and Methods". **b** Effect of *FHL2-siRNA* on the expression of *NF-κB-p65* in DLD1 cells. Fifty microgram of total cell lysate from each sample was loaded in each lane for Western blot. *GAPDH* was used as an internal control

Table 1 Yeast two-hybrid (Y2H) system revealed a potential interaction between FHL2 and NF-κBIα

No. of clones	Gene name	Start code	Stop code	Frame	Orientation	%ID 5p/3p	Global PBS
1	NF-κBIα	12	442	IF	Sense	100/99	C
1	NF-κBIα	117	440	IF	Sense	100/100	C

IF in frame, *Global PBS C* indicates good confidence in the interaction

Yeast Two-Hybrid System Indicates an Interaction Between FHL2 and NF-κBIα

The full-length FHL2 was used as a bait in Y2H screening to identify interacting partners from a human placental cDNA library. Of all clones screened, two clones isolated on the basis of both nutritional selection and β-galactosidase activity contained cDNAs with open reading frames coding for NF-κBIα. As summarized in Table 1, both clones had a global PBS of level C indicating an interaction with good confidence.

In DLD1 cells, knocking down of FHL2 with its siRNA (FHL2-siRNA) suppressed the expression of NF-κB subunit p65 (NF-κB-p65; Fig. 1b).

These results suggest a potential interaction between FHL2 and NF-κB-p65 regulating protein NF-κBIα.

Localization of FHL2 and NF-κBIα in HeLa Cells

We then used HeLa cells as a model to detect whether FHL2 and NF-κBIα are physically co-localized. By immunofluorescent microscopy, exogenous FHL2 was localized in the nucleus of the HeLa cells (Fig. 2a,c), whereas the endogenous NF-κBIα was localized in the cytoplasm (Fig. 2b,d). In separate experiments, the ectopically expressed pEGFP-N1-FHL2 and pDsRed1-N1-NF-κBIα were also found to localize in the nucleus and cytoplasm respectively (data not shown). Similar results were also obtained from studies in DLD1 cells (data not shown).

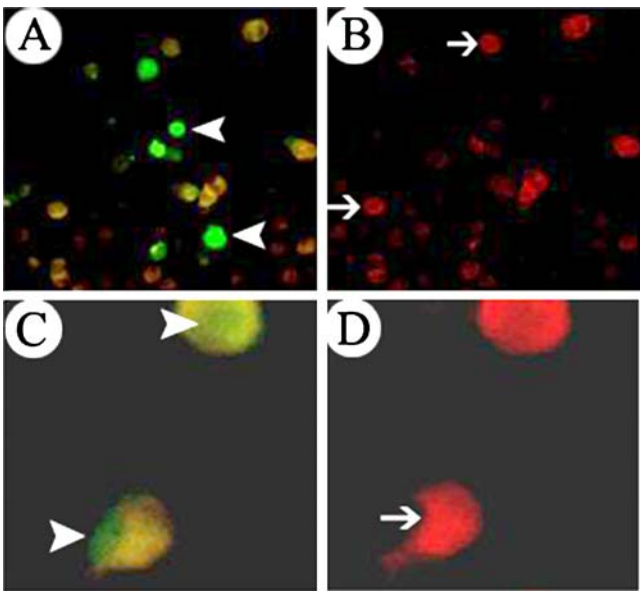

Fig. 2 Localization of FHL2 and NF-κBIα in HeLa cells. Localization of exogenous FHL2 (**a**) and endogenous NF-κBIα (**b**) in HeLa cells by immunofluorescence microscopy. At higher magnification, FHL2 was clearly localized in nucleus (**c**) but NF-κBIα is localized in cytoplasm (**d**)

Co-immunoprecipitation

We used specific antibodies against FHL2 or NF-κBIα to immunoprecipitate the whole cell lysates, followed by probing with anti-NF-κBIα or anti-FHL2. No co-precipitation was observed (Fig. 3). These results indicated that no physical association between NF-κBIα and FHL2 was present in cancer cells.

Discussion

Our group has previous reported that FHL2 plays an important role in gastrointestinal carcinogenesis. [6] However, the mechanism by which FHL2 functions in carcino-

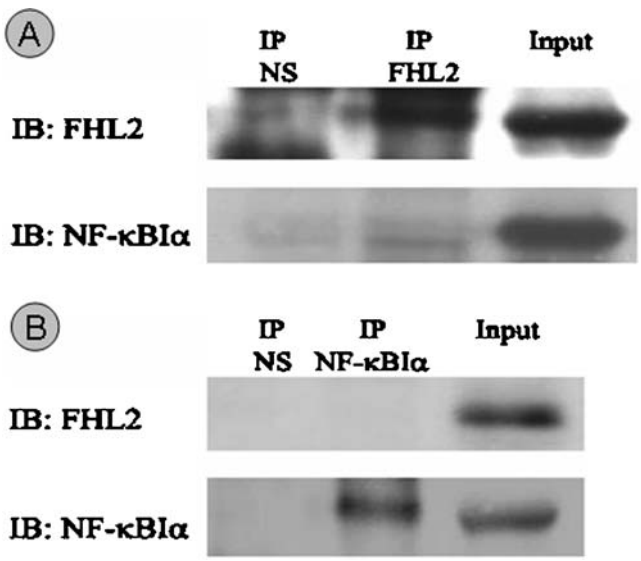

Fig. 3 Co-immunoprecipitation of *FHL2* or *NF-κBIα* in DLD1 cells. DLD1 cells were cultured and processed as described in the "Materials and Methods". Protein lysates precipitated by anti-*FHL2* (**a**) or anti-*NF-κBIα* (**b**) were probed with anti-*FHL2* and anti-*NF-κBIα*

genesis is not fully clarified, especially when the role of FHL2 as an adaptor protein is concerned.

It has been well established that FHL2 serves as an adaptor protein capable of interacting with many transcription factors and thereby inducing various biological functions in different cell types. As NF-κB is such an important transcription factor involved in many aspects of carcinogenesis in many cancers including gastrointestinal cancers, clarification of whether FHL2 interacts with NF-κB would provide useful information on how FHL2 is involved in carcinogenesis and how it can be best utilized as a potential target for the therapy of gastrointestinal cancers.

In the current study, we first identified that both FHL2 and NF-κBIα are richly expressed in the gastric and colon cancer cells, thus forming a prerequisite for further studying their interaction. By using Y2H screening, we identified a potential interaction between FHL2 and NF-κBIα with a level C global PBS. The potential interaction between FHL2 and NF-κBIα was also indicated by the finding that knocking down of FHL2 led to a reduced expression of NF-κB-p65.

We then used two other approaches to confirm this finding: co-localization by fluorescence microscopy and co-immunoprecipitation (Co-IP). These are standard techniques to assess protein-protein interactions.

By co-localization study, we found that both exogenous and endogenous FHL2 and NF-κBIα did not co-localize: FHL2 was localized in the nucleus, whereas NF-κBIα was in the cytoplasm.

By Co-IP study, we attempted to capture FHL2 with its specific antibody and then probed the precipitate for NF-κBIα using a specific antibody against it. A reciprocal Co-IP (i.e., using anti-NF-κBIα to pull down the interaction complex, and then probe for FHL2) was also performed. These approaches, however, did not reveal any interaction between FHL2 and NF-κBIα.

These results demonstrated a lack of physical association between FHL2 and NF-κBIα, and suggested that they are very unlikely to have a direct interaction. However, the fact FHL2-siRNA could down-regulate the expression of NF-κB subunit p65 suggests that under the physiological condition, FHL2 may activate NF-κB pathway, even though such an activation may not be mediated by the direct binding of FHL2 to the NF-κB inhibitor protein IκB.

In conclusion, FHL2 activates NF-κB without direct interaction with NF-κBIα. Further research into the detail mechanism is warranted.

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