Interferon-induced transmembrane protein 1 (IFITM1) is an essential mediator of interferon-γ-induced anti-proliferation. Here, we reported the interaction between IFITM1 and caveolin-1 (CAV-1), and their inhibitory regulatory function on extracellular signal-regulated kinase (ERK). The immunofluorescence staining result showed that IFITM1 localized in caveolae of the plasma membrane and could interact with CAV-1. Deletion mutagenesis clearly revealed that the hydrophobic transmembrane domains were responsible for the interaction between IFITM1 and CAV-1. It has been reported that CAV-1 has inhibitory effect on the phosphorylation of ERK, and subsequently ERK-mediated transcription. Our study showed the interaction of IFITM1- and CAV-1-enhanced CAV-1’s inhibitory effect on ERK activation, whereas the IFITM1 did not activate ERK directly. This inhibitory effect was further confirmed by knocking down the endogenous CAV-1 using RNA interference. These results revealed that the interaction between IFITM1 and CAV-1 could enhance the inhibitory effect of CAV-1 on ERK activation.

Keywords interferon-induced transmembrane protein 1; caveolin-1; ERK; inhibitory effect

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Introduction

Interferon-induced transmembrane proteins (IFITMs) are a family of proteins originally identified as targets of interferon (INF) stimulation in neuroblastoma cells. IFITM1, also known as 9-27 or Leu13, is a cell surface molecule. It is a component of a multimeric complex involved in the transduction of anti-proliferation and cell adhesion signals [1,2]. Human IFITM1 probably plays an important role in the anti-proliferative activity of INFs [3,4].

Extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinases (MAPKs), is essential for cellular proliferation and differentiation. ERK together with c-Jun NH2-terminal kinase (JNK) and Big MAP kinase 1 (also called ERK5) are important signaling components linking extracellular stimuli to cellular responses such as cell growth, death, differentiation, and metabolic regulation [5,6]. The activation of ERK signaling pathway is important for cell proliferation [7,8]. It was reported that ERK was compartmentalized within caveolae [9,10] and CAV-1 could inhibit ERK activity [11–14].

Caveolae is morphologically identifiable as plasma membrane invagination involved in multiple cellular processes, including cholesterol homeostasis, vesicular transport, and the regulation of signal transduction [15]. Caveolae was termed by caveolins, a family of scaffolding proteins. Caveolin-1 (CAV-1) is a highly conserved integral membrane protein. It was reported that the expression of CAV-1 was sufficient and necessary for the formation of morphologically identifiable caveolae [16,17]. CAV-1 might function in the regulation of cell growth, lipid trafficking, endocytosis, and cell migration, and in the activity modulation of multiple signaling molecules such as Scr kinase, epidermal growth factor tyrosine kinase, G protein, and ERK [18,19].

Since both IFITM1 and CAV-1 act as positive regulators of p53, and have inhibitory effects on ERK, it should be interesting to explore the functional relationship between the two proteins. We observed that IFITM1 co-localized with CAV-1 in caveolae of the plasma membrane and interacted with CAV-1 by its hydrophobic transmembrane domains. Furthermore, the interaction between IFITM1 and CAV-1 enhances the inhibitory function of CAV-1 on ERK.
Materials and Methods

Cell culture and transfection
BEL-7404 cells were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). Chang liver cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). These cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum, 100 μg/ml of streptomycin sulfate, and 100 μg/ml of penicillin at 37°C in 5% CO₂. Transient transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol.

Plasmid construction
The coding region sequence of the human IFITM1 gene was amplified from cDNA of Chang liver cells using specific primers and was cloned into pcDNA3.1B (Invitrogen) and pEGFP-N1 vectors (Clontech, Mountain View, CA, USA). In a similar manner, the coding region sequences of human ERK2 and CAV-1 were cloned into a pcDNA3.1B vector. All the gene sequences obtained by PCR were verified by sequencing.

Immunocytochemistry
Chang liver and BEL-7404 cells were cultured on glass slides. Half of the cells were transfected with plasmids of IFITM1-green fluorescent protein (GFP). All the cells were harvested after 24 h treatment of 500 U/ml IFN-γ. Cells were then fixed with 4% paraformaldehyde and permeated by permeabilizing solution (0.1% Triton X-100 and 0.1% sodium citrate in PBS) for 5 min on ice. After being blocked in 3% BSA, the slides were incubated with the primary antibodies of anti-CAV-1 (anti-mouse, 2 μg/ml) (Santa Cruz, Santa Cruz, CA, USA) and/or anti-IFITM1 (anti-rabbit, 2 μg/ml) (Boster, Wuhan, China). After being washed three times with PBS, the slides were incubated with FITC-conjugated anti-mouse IgG (1:200) (Santa Cruz) and Cy5-conjugated anti-rabbit IgG (1:200) (Jackson ImmunoResearch, West Grove, PA, USA). Cells were mounted with Slow-Fade and examined using an Olympus fluorescence microscope.

Sodium carbonate extraction and sucrose density gradient fractionation of lipid rafts/caveolae
The experiments were carried out following the detergent-free protocol developed by Song et al. [20]. Briefly, two 10-cm dishes of monolayer cells were washed with ice-cold PBS, scraped into 2 ml of 0.5 M Na₂CO₃ (pH 11.0), and sonicated. The homogenized cell sample was mixed with an equal volume of 90% sucrose solution in MES-buffered saline (25 mM MES, pH 6.5 and 0.15 M NaCl), placed at the bottom of an ultracentrifuge tube, and overlaid with 4 ml of 35% sucrose and 4 ml of 5% sucrose in MES-buffered saline containing 0.25 M Na₂CO₃. The gradient centrifugation was carried out at 39,000 rpm for 20 h in an SW41 rotor (Beckman, Pasadena, CA, USA). Fractions were collected from the top of the gradient. Proteins were precipitated with 12.5% trichloroacetic acid, dissolved in 1/4 Laemmli SDS sample buffer containing 20 mM dithiothreitol, and used in western blot.

In vivo co-immunoprecipitation
Co-transfected or untransfected Chang liver cells were treated with 500 U/ml IFN-γ for 24 h. Then the cells were lysed for 1 h at 4°C in immunoprecipitation (IP) buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP-40, 0.2 mM Na₃VO₃, and 0.2 mM PMSF). Cell extracts were incubated overnight at 4°C with anti-flag (Wolwo Biotech, Shanghai, China), anti-CAV-1 (Santa Cruz), and anti-IFITM1 (Boster) antibodies (2 μg/ml), respectively. Immuno-complexes were isolated by incubation with protein A agarose (Invitrogen) for 2 h at 4°C. The immunoprecipitated samples were washed extensively with IP buffer and subjected to western blot with the corresponding antibodies.

Synthesis and transfection of siRNA
siRNA sequences targeting human CAV-1 gene (AACCAGAAAGGACACACAG) were synthesized (GenePharma, Shanghai, China) [21]. The sequences targeting GFP gene (GAACGGCATCAAGGTGAAC) were used as a non-specific siRNA control. The siRNA was transfected with Lipofectamine 2000. After 24 h, siRNA-transfected cells were further transfected with IFITM1 and/or CAV-1 plasmids for additional 24 h and harvested for immunoblot analysis.

Determination of ERK activation by western blot analysis
Cells transfected with different plasmids were harvested on ice. Cell lysates were resolved and loaded on 15% SDS polyacrylamide gel. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) and probed with...
antibody specific to phosphorylated forms of ERK (anti-p-ERK; 1:400) (Santa Cruz) to determine the activation status of MAP kinase as described [22]. As a control, the total amount of ERK was determined by western blot analysis using an ERK1 antibody (1:2000) (Santa Cruz) that also cross-reacted with ERK2 [22]. Goat anti-rabbit IgG conjugated to alkaline phosphatase was used as the secondary antibody. Anti-flag (1:10,000) (Wolwo Biotech) and anti-myc (1:4000) (Wolwo Biotech) antibodies were used to detect the proteins expressed. The expression levels of the proteins were quantified by TotalLab 2.0.

**Reporter gene assay**

PathDetect Elk trans-Reporting system vectors (pFR-Luc and pFA-Elk) were purchased from Stratagene (La Jolla, CA, USA). For measuring ERK transfection activation, Chang liver cells were co-transfected with ERK2-pcDNA3.1, pFR-Luc, pFA-Elk, pRL-TK (Promega, Madison, WI, USA), and IFITM1, CAV-1, empty vector plasmid alone or combined and cultured in 1% FBS for 36 h after transfection. Firefly (ff-Luc) and Renilla (r-Luc) luciferase activities in lysates were measured by a dual luciferase assay system (Promega) following the manufacturer’s protocol.

**Results**

**IFITM1 co-localized with CAV-1**

IFITM1 was initially discovered in T89G neuroblastoma cells that expressed the mRNA in response to INF stimulation [23,24]. The localization of endogenous IFITM1 and CAV-1 on cell surface was corroborated via double immunofluorescence staining with mouse anti-CAV-1 and rabbit anti-IFITM1 antibodies. As shown in Fig. 1, the localization of GFP-fused IFITM1 proteins [Fig. 1(A)] and endogenous IFITM1 proteins [Fig. 1(B,C)] was similar to that of endogenous CAV-1.
proteins [Fig. 1(D–F)]. The overlay images showed, in yellow, most of IFITM1 proteins co-localized with CAV-1 proteins [Fig. 1(G–I)]. Also, density gradient fractionation experiment was carried out, aiming to validate the localization of the two proteins. As indicated in Fig. 2, IFITM1 was found to be expressed in the same fractions with CAV-1 in both Chang liver and BEL-7404 cell lines. Caveolins were predominantly found on plasma membrane [25] and could be used as indicator of caveolae on cell surface. Thus, it could be concluded that IFITM1 and CAV-1 co-localized in caveolae of the plasma membrane.

IFITM1 interacted with CAV-1 through their hydrophobic transmembrane domains in vivo
Co-IP assay was performed to further probe the interaction between IFITM1 and CAV-1. In Chang liver cells, flag-tagged IFITM1 was co-transfected with myc-tagged CAV-1 plasmid. Exogenous fag-tagged IFITM1 proteins were co-immunoprecipitated with exogenous myc-tagged CAV-1 proteins [Fig. 3(A)]. Endogenous Co-IP assay shows the similar result [Fig. 3(B)]. After incubation with CAV-1 antibody, the endogenous IFITM1 proteins were co-immunoprecipitated with endogenous CAV-1 proteins, but not the normal IgG control. These results suggested that IFITM1 protein interacted with CAV-1 protein in vivo.

To determine the interaction domains on IFITM1 and CAV-1, we constructed series gene deletion to map the binding domains [Fig. 3(C,E)]. Flag-tagged IFITM1 without residues 84–125 [Fig. 3(D), line 13] and myc-tagged CAV-1 lacking residues 102–178 [Fig. 3(F), line 13] could not be immunoprecipitated. It indicated that IFITM1 interacted with CAV-1 through their hydrophobic transmembrane domains. The second transmembrane domain (residues 83–118) of IFITM1 and the transmembrane domain (residues 102–134) of CAV-1 were both indispensable for the interaction between IFITM1 and CAV-1 proteins.

IFITM1 enhanced the ability of CAV-1 in inhibiting ERK activation
We previously reported that IFITM1 inhibited the activation of ERK [4]. It has been proved that CAV-1 could interact directly with ERK and inhibits the activation of ERK [26]. Considering these facts, further experiments were conducted to confirm whether the interaction between IFITM1 and CAV-1 could affect ERK activation.

Phosphorylation levels of ERK were monitored in BEL-7404 and Chang liver cell lines transfected with CAV-1 and/or IFITM1. As shown in Fig. 4(A,B), the phosphorylation of ERK could be inhibited by both IFITM1 and CAV-1. The level of phosphorylated ERK was greatly decreased by co-transfection of CAV-1 and IFITM1. Using reporter assay, the transfection activity of ERK was detected by measuring Elk activity, the down-stream component of ERK signaling pathway. The result was in agreement with the western blot analysis [Fig. 4(C)]. RNAi ablation of CAV-1 was performed in BEL-7404 and Chang liver cells [Fig. 4(D)]. It was observed that the activation of ERK was not inhibited by IFITM1, whereas endogenous expression of CAV-1 was lost. The result was further supported by western blot [Fig. 4(E)]. The reporter assay monitoring Elk activity was also applied and showed the similar results [Fig. 4(F)]. In this sense, IFITM1 was able to inhibit the activation of ERK via CAV-1, and even enhance the inhibitory function of CAV-1 on ERK activation.

Discussion
IFITM1, a transmembrane protein, has the ability of inhibiting cell growth and tumor genesis. It was reported that IFITM1 localized on ER membrane [26]. In this study, it was found that IFITM1 could localize in...
Caveolae of the plasma membrane. CAV-1, also a transmembrane protein, was found to localize in the plasma-lemma, secretory vesicles, Golgi, mitochondria, ER membrane, and lipid rafts [27–29]. IFITM1 and CAV-1 were partially co-localized in caveolae of the plasma membrane and could interact with each other by their transmembrane domains.

In the experiment, IFITM1 could co-immunoprecipitated by CAV-1 instead of exogenous CAV-1-P132L (proline to leucine substitution), a CAV-1 mutant (data not shown). Furthermore, CAV-P132L does not have the same subcellular location as CAV-1 [30]. Therefore, the interaction between IFITM1 and CAV-1 might rely on the proper localization of CAV-1.

ERK activity is up-regulated in many types of cancers. The intracellular Ras-regulated Raf/MEK/ERK protein kinase signaling cascades play a central role in coordinating cell cycle re-entry, cell survival, cell motility, and invasion in response to virtually all known growth factors. IFITM1 binds CAV-1 and enhances the inhibitory effects of CAV-1 on ERK activation, resulting in negative regulation of the MAPKs signaling pathway. Since CAV-1 is considered as a tumor repressor in many types of cancers, IFITM1 might also restrain tumor

Fig. 3 Interaction between IFITM1 and CAV-1 in Chang liver cells  (A) Exogenous co-IP assay. Chang liver cells were transfected as described in the Materials and Methods section and then used in IP with anti-myc antibody. Co-immunoprecipitated flag-tagged IFITM1 protein was detected with anti-flag antibody. (B) Endogenous co-IP assay. Cell lysates were incubated with anti-CAV-1 antibody or mouse IgG, and immunoprecipitated proteins were detected by anti-IFITM1 antibody. Schematic diagram of the IFITM1 (C) and CAV-1 (D) deletion mutants. TM, transmembrane. (D) Mapping the interaction domains of IFITM1 with CAV-1. Cells were co-transfected with myc-tagged CAV-1 and flag-tagged IFITM1 deletion mutants and then subjected to IP with anti-myc antibody. Co-immunoprecipitated flag-tagged IFITM1 deletion mutants were detected with anti-flag antibody. (F) Mapping the interaction domains of CAV-1 with IFITM1. Cells were co-transfected with flag-tagged IFITM1 and myc-tagged CAV-1 deletion mutants and then subjected to IP with anti-myc antibody. Co-immunoprecipitated flag-tagged IFITM1 deletion mutants were detected with anti-flag antibody.
formation or growth, which would be promising for further investigation.

The inhibiting function of IFITM1 on ERK activation has been investigated in our previous work. In this study, it was found that the inhibition was mediated by CAV-1, and IFITM1 could further enhance the inhibiting function of CAV-1 on ERK. Since IFITM1 could interact with CAV-1, and they function similarly on regulating cell growth, we hypothesized that IFITM1 and CAV-1 could form a protein complex to regulate the cell growth and tumor genesis.

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Fig. 4 IFITM1 enhanced the ability of CAV-1 in inhibiting ERK activation through the interaction with CAV-1 The phosphorylation levels of ERK in Chang liver cells (A) and BEL-7404 cells (B). Cells were transfected as indicated. Cell lysates were detected by anti-p-ERK, anti-ERK, anti-myc, and anti-flag antibodies, respectively. The expression level of the proteins was measured by Total Lab software as mentioned in the Materials and Methods section, and the results were shown as the mean ± SD of three independent experiments. (C) The luciferase activity in Chang liver cells. Cells were transfected as indicated. (D) CAV-1-siRNA inhibited endogenous expression of CAV-1 gene in Chang liver and BEL-7404 cells. Cells were infected with siRNA targeting CAV-1 and control for 48 h. The cell lysates were blotted with anti-CAV-1 antibody. Actin was shown as a loading control. (E) The phosphorylation levels of ERK in Chang liver and BEL-7404 cells. The siRNA was transfected with Lipofectamine 2000. After 24 h, siRNA-transfected cells were further transfected with IFITM1 and/or CAV-1 plasmids for additional 24 h and harvested for immunoblot analysis. Cell lysates were detected by anti-p-ERK and anti-ERK antibodies, respectively. The expression level of the proteins was measured by Total Lab, and the results were the mean ± SD of three independent experiments. (F) The luciferase activity in Chang liver cells. Luciferase activity was measured after 48 h transfection. Results were the mean ± SD of three independent experiments.
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