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Human Intersectin 2 (ITSN2) binds to Eps8 protein and enhances its degradation

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Participates in actin remodeling through Rac and receptor endocytosis via Rab5. Here, we used yeast two-hybrid system with Eps8 as bait to screen a human brain cDNA library. ITSN2 was identified as the novel binding factor of Eps8. The interaction between ITSN2 and Eps8 was demonstrated by the *in vivo* co-immunoprecipitation and colocalization assays and the *in vitro* GST pull-down assays. Furthermore, we mapped the interaction domains to the region between amino acids 260-306 of Eps8 and the coiled-coil domain of ITSN2. In addition, protein stability assays and immunofluorescence analysis showed ITSN2 overexpression induced the degradation of Eps8 proteins, which was markedly alleviated with the lysosome inhibitor NH4Cl treatment. Taken together, our results suggested ITSN2 interacts with Eps8 and stimulates the degradation of Eps8 proteins. [BMB reports 2012; 45(3): 183-188]

INTRODUCTION

Eps8, originally identified as a substrate for the epidermal growth factor receptor kinase, acts as a multifunctional molecule involved in EGFR signaling (1, 2) and the development of certain malignancies (3-5). The functions of Eps8 have been extensively studied by its domains. The N-terminal phosphotyrosine binding domain (PTB) is a conserved protein-protein interaction domain in a phosphotyrosine-dependent and -independent fashion (6-10). The proline-rich region (PR) is involved in IRSp53/Eps8 complex formation (11). The EGFR binding region (BR) of Eps8 is required for the binding of the juxtamembrane region of EGFR and enhances EGF-dependent mitogenic signals (2). The Src-homology-3 (SH3) domain is a ubiquitous interaction module (12, 13), two interactors, E3b1/Abi-1 and RN-tre, bind to the SH3 domain of Eps8 and regulate cell growth (14, 15). Further studies showed the Rab5 GTPase-activating protein RN-tre binds with Eps8 to inhibit internalization of the EGFR and control intracellular membrane trafficking (16). The Eps8 C-terminal effector region is responsible for the interaction with Sos1 and F-actin (17). Moreover, Abi-1 bridges Eps8 and Sos1 to form a tricomplex and the complex induces Rac-specific guanine nucleotide exchange factor (GEF) activity, transduces signals from Ras to Rac, enhances Rac-dependent actin remodeling (17-19). Therefore, the Eps8 protein coordinates EGF receptor signaling through Rac and endocytic trafficking through Rab5.

Eps8 was implicated in many tumor cell proliferation and invasion. High expression and concomitant tyrosine phosphorylation of Eps8 were detected in tumor cell lines (20). Eps8 enhances EGF-dependent mitogenic signals and leads to malignant transformation (1, 20). Eps8 increases cell growth and motility, by the up-regulation of FOXM1, FAK, MMP-9, ERK, Akt, cyclins D1, D3, and E, the down-regulation of P53 and p21Waf1/Cip1 in squamous cell carcinoma, pancreatic cancer, cervical cancer, colon cancer, pituitary tumors and esophageal carcinomas (4, 5, 21-26).

Eps8 is an important signal molecule, we demonstrated here that ITSN2 and Eps8 interact *in vivo*, and the interaction between both proteins results in the decrease of Eps8 proteins.

RESULTS

ITSN2 interacts with Eps8 in yeast two-hybrid screening

To identify Eps8-interacting proteins, we performed yeast two-hybrid screen. Yeast strain MaV203 was transfected with bait plasmid pDBLeu-Eps8 and then prey plasmid pPC86-cDNA with human brain cDNA library cloned with the GAL4 activation domain of pPC86 vector. After screening on a SD-Leu$^-$, Trp$^-$, uracil$^-$, His$^-$ medium supplemented with 25 mM 3-AT, ITSN2 was identified as an interacting partner of Eps8 by X-galactosidase filter assays and sequencing analysis (Fig. 1A). Likewise, blue colonies appear in positive control C and positive control D but not in controls expressing pDBLeu-Eps8 and

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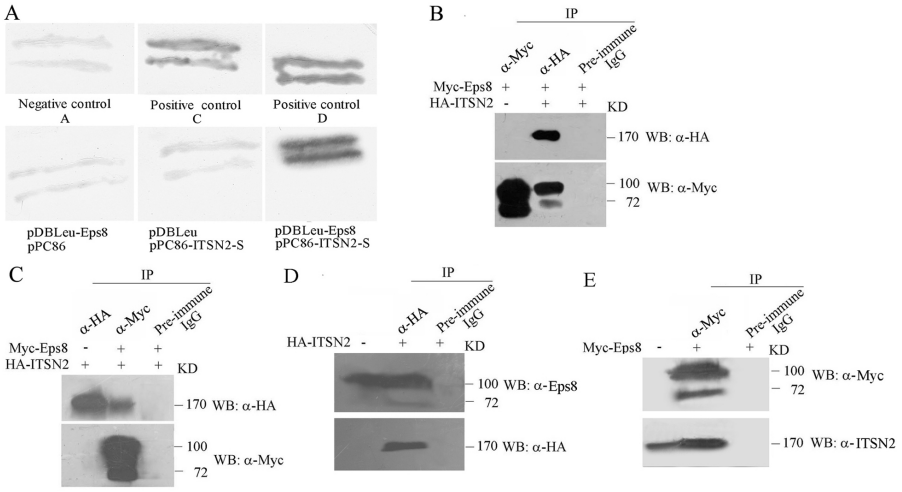

Fig. 1. Interaction between ITSN2 and Eps8 *in vivo* by yeast two-hybrid system and co-immunoprecipitation assays. (A) The indicated vectors were co-transformed into the yeast MaV203 cells and analyzed for the reporter activity of lacZ using X-galactosidase filter assays. Three controls are set. (B-E) HEK293FT cells were transfected with the indicated plasmids and harvested 30 h after transfection. Cell extracts were incubated with the anti-Myc or anti-HA antibodies, precipitated by ProteinA/G beads and detected with the indicated antibodies. Pre-immune IgG was used as negative control.

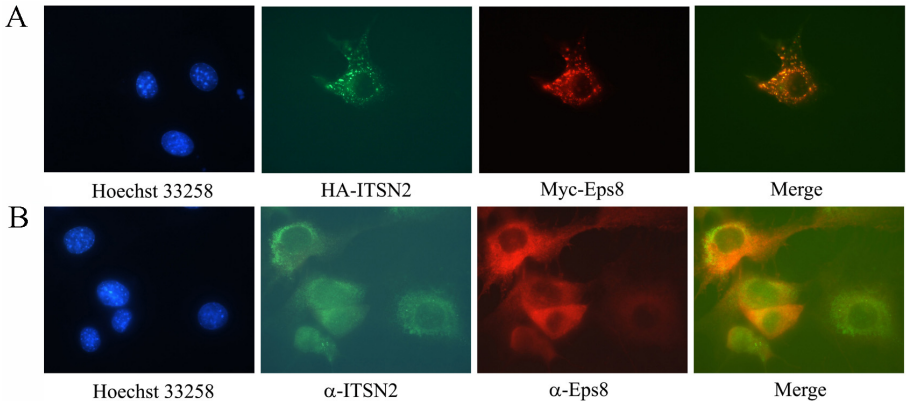

Fig. 2. Co-localization assays of ITSN2 and Eps8 proteins in HEK293 cells. (A) HEK293 cells were co- transfected with HA-ITSN2 and Myc-Eps8. HA-ITSN2 was detected with anti-HA antibodies, while Myc-Eps8 was detected with anti-Myc antibodies. (B) Endogenous ITSN2 was detected with rabbit polyclonal anti-ITSN2 antibodies, while endogenous Eps8 was detected with mouse monoclonal anti-Eps8 antibodies.

pPC86, pDBLeu and pPC86-ITSN2-S, or negative control.

ITSN2 and Eps8 are co-immunoprecipitated in HEK293FT cells

To further demonstrate whether ITSN2 interacts with Eps8 *in vivo*, HEK297FT cells were transiently transfected with expression plasmids pCMV-Myc-Eps8 and pCMV-HA-ITSN2 or pCMV-Myc-Eps8 or pCMV-HA-ITSN2. The lysates were immunoprecipitated with the indicated antibodies, and the co-immunoprecipitated proteins were analyzed. Eps8 was detected in immune complexes of HA-ITSN2, but control IgG did not precipitate any band (Fig. 1B). Likewise, ITSN2 was detected in immunoprecipitates of Myc-Eps8, whereas preimmune IgG did not recognize target protein (Fig. 1C). Moreover, ITSN2 interacts with not only 97KD proteins of Eps8 main isoform (Eps8-L), but also 68 KD proteins of Eps8 short isoform (Eps8-S), the proteolytic or alternatively spliced product of the 97 KD Eps8 isoform (1, 27). Further results showed that endogenous Eps8 was detected in immune complexes of HA-ITSN2 (Fig. 1D), whereas endogenous ITSN2 was detected in immunoprecipitates of Myc-Eps8 (Fig. 1E). Therefore, these data clearly suggested that ITSN2 interacts with Eps8 *in vivo*.

ITSN2 and Eps8 are co-localized in the cytoplasm

Next, we investigated whether ITSN2 and Eps8 are co-localized in the same cellular structure *in vivo*. We found HA-ITSN2 was co-localized with Myc-Eps8 in the cytoplasm of HEK293 cells (Fig. 2A). Moreover, endogenous ITSN2 and Eps8 proteins were significantly localized in the cytoplasm, and endogenous ITSN2 proteins completely merged with endogenous Eps8 proteins (Fig. 2B). These data indicated that ITSN2 and Eps8 exist in the same complex *in vivo*.

ITSN2 and Eps8 directly interact *in vitro*

Since overexpressed ITSN2 and Eps8 could interact *in vivo*, we wonder whether ITSN2 directly interacts with Eps8 *in vitro*. The truncated Eps8 (Fig. 3A) were bacterially expressed as GST recombinant proteins and purified (Fig. 3B), while truncated ITSN2 (Fig. 3A) (28) were bacterially expressed as His fusion proteins and purified (Fig. 3B). GST pull-down assays showed

His-ITSN2-S (screened clone by Eps8) specifically bound to GST-Eps8-1 (1-306 a.a.), but not to other two Eps8 truncated proteins or GST. Based on the structure differences of Eps8 two isoforms (Fig. 3A), the 68 KD Eps8-S isoform did not include the PTB domain and proline-rich region encompassing 1-260 amino acids, demonstrating the region comprising amino acids 260 and 306 is necessary for the binding of Eps8 to ITSN2 (Fig. 3C). The domain of ITSN2 interacting with Eps8 was also mapped, we found that His-ITSN2-CC bound to GST-Eps8-1, but not to GST, while His-ITSN2-EH did not bind to either GST-Eps8-1 or GST (Fig. 3D). Thus, amino acids 260-306 of Eps8 and the coiled-coil domain of ITSN2 are critical for their interactions.

ITSN2 enhances the degradation of Eps8 proteins by the lysosome pathway

To further investigate the physiological importance of the

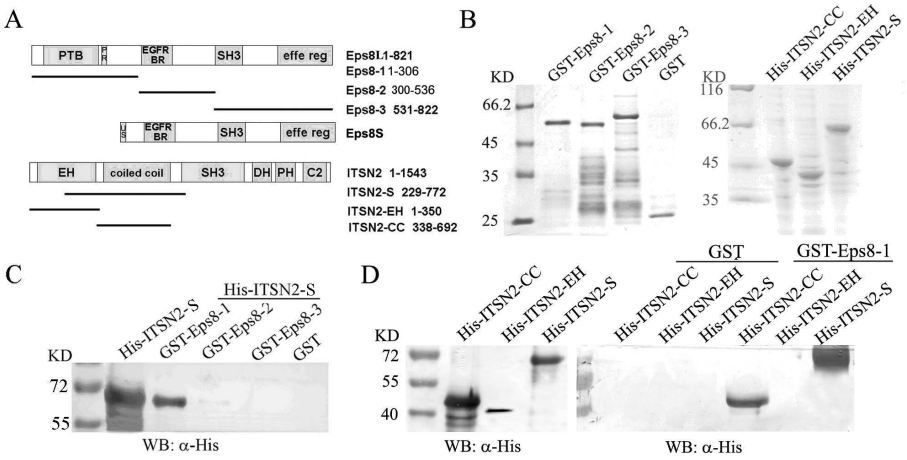

Fig. 3. Interaction between ITSN2 and Eps8 *in vitro* by pull-down assays. (A) Schematic domains of Eps8 and ITSN2. PTB (60-197 a.a.); PR (207-221 a.a.); EGFR BR (296-362 a.a.); SH3 (535-586 a.a.); effe reg, (648-821 a.a.); US, unique sequence; EH, Eps15 homology; DH, Dbl homology; PH, pleckstrin homology; C2, a Ca(2+)-binding motif. (B) The bacterially expressed GST-Eps8 and His-ITSN2 truncated proteins were purified and run on 12% SDS-page. (C) pull-down experiments were performed with GST or GST-Eps8 truncations and His-ITSN2-S incubated with glutathione-Sepharose beads. Bound proteins were resolved by 10% SDS-PAGE gel, and immunoblotted with the anti-His antibodies. (D) The same pull-down assays were performed with GST or GST-Eps8-1 and His-ITSN2 truncations.

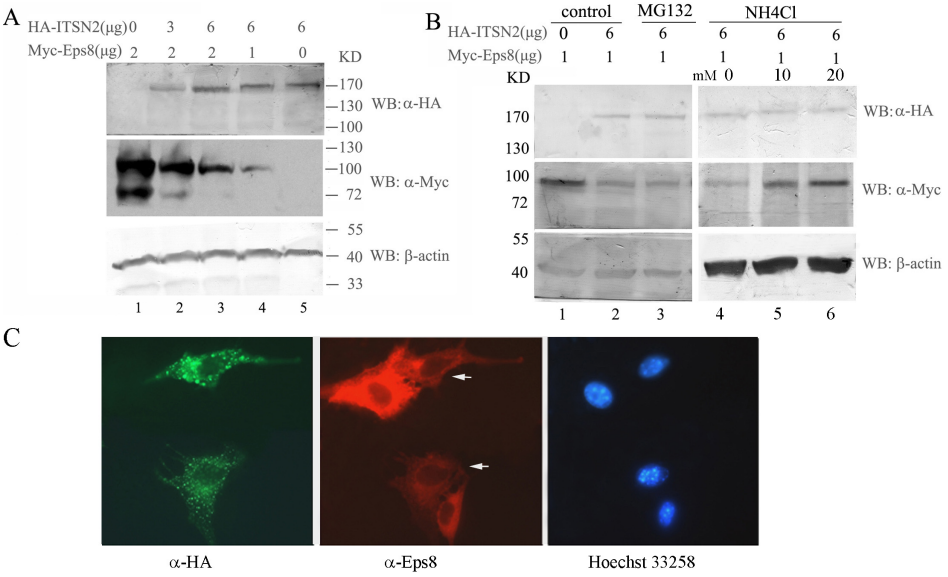

Fig. 4. The effect of the interaction between ITSN2 and Eps8 on protein stability. (A and B) HEK293FT cells were transfected with the indicated expression vectors and transfected cells were treated with 10 mM of MG132 or 10-20 μM of NH4Cl for 10 h. Cell lysates were detected with mouse monoclonal antibodies against HA-tag and Myc-tag. β-actin was used as a loading control. (C) HEK293 cells were transfected with HA-ITSN2 followed by immunofluorescence analysis. HA-ITSN2 was detected with rabbit polyclonal anti-HA antibodies, while endogenous Eps8 was detected with mouse monoclonal anti-Eps8 antibodies. Arrows indicate ITSN2 overexpression induced the decrease of Eps8 proteins.

Eps8-ITSN2 interaction, we performed protein degradation assays. As shown in Fig. 4A, an increase in the amount of ITSN2 proteins induced a significant decrease in Eps8 proteins even though the amount of transfected Eps8 is the same, while the increase of Eps8 proteins kept ITSN2 proteins equal. Likely, immunofluorescence assays showed overexpression of ITSN2 remarkedly reduced the amount of Eps8 proteins (Fig. 4C). These data strongly suggested ITSN2 enhances the degradation of Eps8 proteins.

We next examined Eps8 protein degradation pathway. As shown in Fig. 4B, different concentrations of the lysosomal inhibitor, NH4Cl, rescued the ITSN2-induced degradation of Eps8 proteins. But the proteasome inhibitor, MG132, had no effect on the expression level of Eps8. Thus, it is likely that ITSN2 modulates the degradation of Eps8 proteins by the lysosome pathway.

DISCUSSION

In the present study we identified several candidates that associated with Eps8 in yeast two-hybrid assays. We selected ITSN2 for the further analysis due to two reasons. Firstly, Eps8 bound with F-actin and localized to proper sites of actin remodeling (17, 18), where Abi-1 and Sos-1 also exist (29). The trimeric complex Eps8-Abi-1-Sos-1 could activate a Rac-specific catalytic ability, and regulate actin dynamics (17, 30). Similarly, ITSN2 belongs to the ITSN family consisting of ITSN1 and ITSN2. They share highly similar structures and functions (31). ITSN1 was involved in mitogenic signaling and regulated GTPase cascades, such as Cdc42 (32-35). Furthermore, ITSN1 and Sos1 formed complexes to lead to Ras activation (32). ITSN2 functions cooperatively with WASP and Cdc42 to mediate actin polymerization (36). Secondly, Eps8 participates to EGF receptor endocytosis through Rab5. ITSN2 also plays integral roles in clathrin-mediated endocytosis (28). Thus, ITSN2 functions as one of the signal transducers in actin dynamics and endocytosis. It is likely that ITSN2 interacts with Eps8 and plays roles in cellular functions.

Evidence presented here demonstrated ITSN2 interacts with Eps8 by the *in vivo* and *in vitro* experiments. And ITSN2 and Eps8 are localized in the same cytoplasmic structures. Moreover, ITSN2 only binds to the region comprising amino acids 260 and 306 of Eps8. The special region is required for the interaction between Eps8 and ITSN2. The coiled-coil domain usually serves as structural elements in many proteins and participates in protein-protein interactions and recognitions (37). Via the coiled-coil domain, ITSN2 interacts with Eps8.

Further data revealed that ITSN2 enhances the degradation of Eps8 proteins. Protein degradation includes two major pathways: one is lysosome pathway, the other is proteosome pathway. The lysosomal inhibitor, NH4Cl, block Eps8 degradation, but not the proteasome inhibitor, MG132. Cytosolic proteins could be degraded by lysosomes. Especially, targeting proteins are components of all known substrate proteins for lysosome pathway. The substrate proteins bind to receptors in the organelle membranes, and are transported in the import pathways (38). Based on these data, the cytosolic protein Eps8 is a substrate protein of EGFR, this association between ITSN2 and Eps8 could result in lysosomal targeting of Eps8 and induce Eps8 down-regulation. ITSN2 enhances the degradation of Eps8, which may restrict Rac-specific catalytic activity of the Eps8-Abi1-Sos1 complex and regulate actin dynamics in a certain environment.

In summary, our results present a novel role of ITSN2 to enhance the degradation of EGFR substrate Eps8. What is the physiological function of the interaction between both proteins. ITSN2 is involved in cytoskeletal-dependent cellular movement and receptor degradation, while Eps8 regulates actin dynamics. They interact with the cytoskeleton. We speculate that ITSN2 might serve as a tumor suppressor or as an intermedial mediator of signal pathways in cellular motility and carcinogenesis.

MATERIALS AND METHODS

Plasmid constructs

The full-length cDNA of human Eps8 was inserted in frame with the GAL4 DNA-binding domain of pDBLeu vector to generate pDBLeu-Eps8. The pCMV-Myc-Eps8 fusion plasmid was described previously (39). The full-length cDNA of human ITSN2 was amplified by PCR assembly of three sequential overlapping gene fragments and ligated into pCMV-HA vector to form pCMV-HA-ITSN2 expression vector. The full-length (1-821 a.a.) and subdomains (1-306 a.a., 300-536 a.a., 531-821 a.a.) of Eps8 were inserted into pGEX-4T-2, respectively. Truncations (229-772 a.a., 1-350 a.a. and 338-692 a.a.) of ITSN2 were introduced into pQE-N3. All constructs have been sequenced for verification.

Yeast two-hybrid

Yeast two-hybrid screen was carried out as described previously (40). pDBLeu-Eps8 was used as bait and pPC86-human brain cDNA library was used as prey. pDBLeu-Eps8 and human brain cDNA library were sequentially transformed into yeast strain MaV203, positive clones were grown on SD-leu-, Trp-, Ura-, His- medium supplemented with 25 mM 3-amino-1,2,4-triazole (3-AT) and further confirmed by X-galactosidase filter assays. Finally, plasmids from positive clones were sequenced.

Cell culture and DNA transfection

HEK293FT and HEK293 cells were cultured in DMEM (Gibco) with 10% FBS, penicillin, and streptomycin. Expression plasmids were transfected into cells using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogene).

The *in vivo* co-immunoprecipitation

HEK297FT cells were grown to 90% confluence in 10 cm dishes and co-transfected with the indicated plasmids. After 30 h, HEK293FT cells were harvested and lysed as described previously (40). The lysates were precipitated using either rabbit

polyclonal antibodies against Myc-tag or HA-tag (Santa Cruz Biotech), immunoprecipitation was separated on 10% SDS-polyacrylamide gel and detected by western blotting.

Immunofluorescence assay

HEK293 cells were grown to 70% confluence on glass coverslips, and cotransfected with pCMV-Myc-Eps8 and pCMV-HA-ITSN2. 24 h later, cells were treated as described previously (40). The primary antibodies used were mouse monoclonal anti-Myc antibodies and rabbit polyclonal anti-HA antibodies while the secondary antibodies were Alexa 594 goat anti-mouse antibodies and Alexa 488 goat anti-rabbit antibodies (Molecular Probes). Endogenous Eps8 proteins were recognized by mouse monoclonal anti-Eps8 antibodies (BD clontech), while endogenous ITSN2 proteins were recognized by rabbit polyclonal anti-ITSN2 antibodies generated by immunizing a healthy male rabbit with GST fusion proteins encompassing amino acids 229-772 of ITSN2. The nucleus was stained with Hoechst 33258 (Sigma). Fluorescent signals were analyzed using a fluorescence microscope (Zeiss Axioskop 2).

The *in vitro* pull-down assays

GST recombinant proteins and His fusion proteins were expressed and purified according to manufacturer's protocols (Amersham). 5 μg of GST or GST fusion proteins were mixed with glutathione-Sepharose 4B beads for 30 min in binding buffer (40). Then 5 μg of His fusion proteins were added to incubate for 2 h. The proteins bound to beads were washed, boiled and resolved by 10% SDS-polyacrylamide gels. These proteins were analyzed by western blotting with mouse monoclonal antibodies against GST (Santa Cruz Biotech) and His-tag (Clontech).

Protein degradation assays

HEK293FT cells were transfected with different amounts of plasmid pCMV-Myc-Eps8 and pCMV-HA-ITSN2. For the identification of degradation pathway, transfected HEK293FT cells were treated with 10 μM of MG132, a specific proteasome inhibitor, or different concentrations of lysosome inhibitor NH4Cl for 10 h. After 30 h, cells were collected and lysed in the RIPA buffer. The lysates were separated by 10% SDS-polyacrylamide gels. Followed by Western blotting.

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