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Identification of ITGB4BP as a new interaction protein of P311

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ABSTRACT

Aims: P311 is an 8 kDa protein that has been shown to be of importance in the process of myofibroblast transformation, glioblastoma invasion and nerve regeneration. However, the interaction protein of P311 has yet to be found. The purpose of this study was to find the interactive protein of P311.
Main methods: The yeast two-hybrid system was used for screening the potential interaction proteins of P311. Joint expression of the potential interactive protein and P311 was immunohistochemically stained. The interaction between P311 and the selected protein was further confirmed by fluorescence resonance energy transfer (FRET) in pulmonary adenocarcinoma tissue sections, and by coimmunoprecipitation in HEK293.
Key findings: Integrin β4 binding protein (ITGB4BP) was confirmed as the interaction protein of P311. Co-expression and interaction of ITGB4BP and P311 were demonstrated in pulmonary adenocarcinoma by both immunohistochemistry and FRET. Moreover the interaction between P311 and ITGB4BP was demonstrated by coimmunoprecipitation in HEK293.
Significance: The interactions between P311 and ITGB4BP may be very important in the process of tumor cell differentiation and metastasis. ITGB4BP may provide a potential new target for the therapy of tumors.

Introduction

P311, also referred to as pentylenetetrazol (PTZ)-17 or c5orf13, is an 8-Kda intra-cytoplasmic protein that is found highly expressed in the germinal zones and superficial cortical layers at embryonic days 17 and 20, respectively, and in the granular layer of the cerebellum, the hippocampus, and the olfactory bulb of the adult mouse (Studler et al., 1993). It does not belong to any known family of proteins, and its biological function has not been fully determined. The expression of P311 is also tightly regulated during the critical period of alveolar formation. Under some pathological conditions, the absence or low expression of P311 may contribute to the failure of alveolar regeneration and lead to the development of human emphysema (Zhao et al., 2006), and the high expression of P311 may be involved in the hypertrophic scar formation (Tan et al., 2010). There are also some evidences showing that the expression of P311 in small-cell lung carcinoma (SCLC) and large-cell neuroendocrine carcinoma (LCNEC) is higher than that in other tumors or normal lung tissue (Jones et al., 2004).

However, P311's interactive protein partner has yet to be identified. In the present study, a yeast two-hybrid system, immunofluorescent staining, fluorescence resonance energy transfer (FRET), coimmunoprecipitation and gene transferring have been used to identify the possible interaction(s) between P311 and its partner.

Materials and methods

Yeast two-hybrid screening

Yeast two-hybrid screening was carried out as described previously (Jung et al., 2006). Briefly, the full length of the coding region of P311 (a kind gift from Prof. Matthieu Levi-Strauss) was inserted in-frame into the multiple cloning sites of the DNA-BD vector, pGBKT7 (Clontech, Palo Alto, CA), to generate the bait plasmid pGBKT7-P311, which was subsequently confirmed by sequencing. The pGBKT7-P311 was transformed into the bait strain AH109 (BD Biosciences Clontech). Before screening, the toxicity of the bait protein on the host strain was tested by comparing the growth rate of cells transformed with pGBKT7 and that of cells transformed with pGBKT7-P311. We also tested whether the bait alone would activate reporter

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genes by means of growth of bait transformants on yeast culture plates of SD/-Trp, and SD/-Trp/-Leu/. P311 bait strain AH109 was mated with the pre-transformed Y187/pACT2 human adult liver cDNA library according to the Clontech protocol.

Immunohistochemistry

All the protocols were approved by the Ethic Committee of Southwest Hospital, Chongqing. Immunohistochemical staining was performed as described elsewhere (Wu et al., 2004). Briefly, a pulmonary carcinoma tissue microarray (Department of Pathology, Southwest Hospital, China), containing 25 squamous cell carcinomas, 32 adenocarcinomas, and 4 normal tissues, was reviewed by the same pathologist (B.X.). Paraffin sections (4 μm) were deparaffinized in xylene and rehydrated in a graded series of ethanol, and processed for blocking endogenous peroxidase activity by incubation in 0.3% H_2O_2–methanol in phosphate-buffered saline (PBS). Antigens were retrieved by 0.1% trypsin enzyme digestion. Nonspecific binding was blocked by 1% goat serum albumin in PBS. The sections were incubated either with rabbit polyclonal antibody against human P311 (working dilution 1:200, Beijing Boisynthesis Biotechnology Co., Ltd., China) or mouse polyclonal antibody against human ITGB4BP (working dilution 1:200, Abnova, Taiwan) in 1% BSA in PBS overnight at 4 °C, followed by incubation with a biotinylated anti-rabbit or anti-mouse IgG antibody and horseradish peroxidase-conjugated streptavidin (SP kit, Zhongshan, China), and finally reacted with 3,3′-diaminobenzidine. Each section was counterstained with hematoxylin then mounted with neutral balsam. To avoid being misled by border staining, these areas were excluded from evaluation. The expression of P311 and ITGB4BP was scored under the microscope as negative (−) or positive (+). The evaluation was done independently by two pathologists (B.X. and Y.S.) on a total of 61 core biopsies; only tissues with complete agreement in their scores were used for subsequent analysis.

Indirect immunofluorescence and FRET analysis

FRET analysis (Konig et al., 2006; Nishi et al., 2004) was used to determine the likely possibility of interactions between P311 and ITGB4BP. Briefly, pulmonary carcinoma sections were incubated with primary antibodies followed by incubation with the corresponding Cy3-conjugated goat anti-rabbit IgG or Cy5-conjugated goat anti-mouse IgG antibody (1:100, Beyotime, China). DAPI was used for DNA staining. After extensive washing of unbound antibodies with PBS, the sections were mounted using anti-fade mounting medium. For double immunofluorescent analyses, sections were incubated overnight at 4 °C with both primary antibodies simultaneously, followed by incubation with both corresponding secondary antibodies, DAPI staining and then mounted. All images and image stacks were collected using a Zeiss LSM 510 META mounted on an Axiovert 200M inverted microscope with a 63×/1.4 NA Zeiss Plan-Apochromat oil-immersion objective. Protein–protein interaction between Cy3-labeled P311 and Cy5-labeled ITGB4BP was studied with a FRET microscope by emission fingerprinting plus acceptor photobleaching on pulmonary carcinoma tissue sections. All images were 512×512 pixels in size and had a 12-bit pixel depth and were obtained under the same scanning conditions. First, single spectral signatures of Cy3/Cy5 within the specimen were captured by means of lambda stack acquisition with corresponding excitation at 543/633 nm and the signals then detected at 10 nm intervals from 550 through 730 nm using an HFT 488/543/643 dichroic mirror. This was followed by normalization of fluorescence emission intensity between 550 nm and 730 nm. Acceptor (Cy5) photobleaching was performed by irradiation of Cy5 in four regions of interest 800 times with the 633 nm laser line set at the intensity of 89.2% with a line average of four. The images were then subjected to emission spectral analysis in order to detect the changes of fluorescence intensity of the donor molecule (Cy3) before and after photobleaching of the acceptor molecules. The FRET efficiency was calculated using the formula: FRETeff = (Ipost − Ipre)/Ipost (equation 1), where FRETeff is FRET efficiency; Ipost is the donor (Cy3) fluorescence intensity after Cy5 photobleaching, and Ipre is the donor (Cy3) fluorescence intensity before Cy5 photobleaching. We analyzed four areas in each specimen from three independent experiments. Identical calculations were performed in selected regions of the images that contained no bleached cells as the control. The calculated FRETeff was further used to estimate the intermolecular distance (R) between the donor and the acceptor according to the formula: $FRETeff = 1/(1+(R/R0)^6)$ (equation 2), where R0, the distance for which a 50% FRET efficiency occurs, is known to be 50 Å for the Cy3/Cy5 FRET pair (Koopmans et al., 2007).

Confocal microscopy analysis

A Zeiss LSM 510 microscope was used for the determination of colocalization of P311 and ITGB4BP in molecules at the few hundred nanometer level. HEK (Human embryonic kidney) 293 cells were grown on coverslips in 6-well plates with DMEM media (Hyclone, USA) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, USA). HEK293 cells were then cotransfected with expression adenovirus encoding EGFP-ITGB4BP and DsRed-p311 under the condition of Lipofectamine reagent (Invitrogen, USA), 8 μl per 1 μg of DNA. Forty-eight hours post-transfection, HEK 293 cells on coverslips were sealed with glycerol and viewed under a confocal microscope with fluorescence excitation intensity at both 488 nm (green fluorescence) and 543 nm (red fluorescence).

Immunoprecipitation and immunoblot analysis

HEK293 cells were grown in DMEM media (Hyclone, USA) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, USA). Transfections were performed in 6-well plates either using Lipofectamine reagent (Invitrogen, USA), 8 μl per 1 μg of DNA. Forty-eight h following transfection, HEK 293 cells were washed with ice-cold PBS phosphate-buffered saline), and lysed in RIPA lysis buffer (4 ml RIPA containing 100 μl 10 mg/ml PMSF, 400 μl protease inhibitor cocktail, 500 μl 100 μM Lactacystin, 10 μl 0.1 g/ml o-phenanthroline) (Santa Cruz, USA). Lysis was allowed to continue for 30 min on ice and was then spun down at full speed at 16,000 rpm for 1 min. Some lysate representing the total lysate was removed and boiled with SDS loading buffer, while the rest was first pre-cleared with rabbit IgG (Boster, China) for 2 h at 4 °C, followed by incubation with Protein A/G beads (Boster, China) for 2 h at 4 °C. The supernatant was divided into two parts, and incubated separately with 1 μg rabbit anti-P311 polyclonal antibody (Bioss, China) and rabbit anti-EGFP polyclonal antibody (Bioss, China) for 2 h at 4 °C. Then Protein A/G beads (boster, China) were added into the immune complex for incubation overnight at 4 °C. The beads were washed three times with RIPA lysis buffer and boiled with SDS loading buffer. The proteins were separated by SDS-PAGE and blotted

Fig. 1. The screening of the potential interactive proteins of P311 by yeast two-hybrid system. We conducted a yeast two-hybrid system for screening a human adult liver cDNA library using human P311 as bait. (A) After four rounds of screening of 1.75×10^7 diploid colony-forming units, 98 colonies were grown on SD/-Ade/-His/-Leu/-Trp (QDO) plates and showed positive phenotypes on QDO/X-a-gal plates. (B) 45 plasmids were isolated from positive colonies by yeast mini-prep method and 19 independent gene products were reproducibly shown to be positive by sequencing analysis of the amplified inserts and BLAST search. Sequencing and database analysis indicated that Prey-47 shared ~99% sequence identity with human integrin beta 4 binding protein (ITGB4BP) (GenBank accession no. NM002212).