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ORIGINAL

Importin α-importin β complex mediated nuclear translocation of insulin-like growth factor binding protein-5

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Abstract. Insulin-like growth factor-binding protein (IGFBP)-5 is a secreted protein that binds to IGFs and modulates IGF actions, as well as regulates cell proliferation, migration, and apoptosis independent of IGF. Proper cellular localization is critical for the effective function of most signaling molecules. In previous studies, we have shown that the nuclear IGFBP-5 comes from ER-cytosol retro-translocation. In this study, we further investigated the pathway mediating IGFBP-5 nuclear import after it retro-translocation. Importin-α5 was identified as an IGFBP-5-interacting protein with a yeast two-hybrid system, and its interaction with IGFBP-5 was further confirmed by GST pull down and co-immunoprecipitation. Binding affinity of IGFBP-5 and importins were determined by surface plasmon resonance (IGFBP-5/importin-β: K_D=2.44e-7, IGFBP-5/importin-α5: K_D=3.4e-7). Blocking the importin-α5/importin-β nuclear import pathway using SiRNA or dominant negative impotin-β dramatically inhibited IGFBP-5-EGFP nuclear import, though importin-α5 overexpress does not affect IGFBP-5 nuclear import. Furthermore, nuclear IGFBP-5 was quantified using luciferase report assay. When deleted the IGFBP-5 nuclear localization sequence (NLS), IGFBP-$5_{\Delta NLS}$ loss the ability to translocate into the nucleus and accumulation of IGFBP-$5_{\Delta NLS}$ was visualized in the cytosol. Altogether, our findings provide a substantially evidence showed that the IGFBP-5 nuclear import is mediated by importin-α/importin-β complex, and NLS is critical domain in IGFBP-5 nuclear translocation.

Key words: Insulin-like growth factor-binding protein, Importin, Nuclear localization signal, Nuclear translocation

INSULIN-LIKE GROWTH FACTOR (IGF) binding protein-5 (IGFBP-5) is one of a family of six structurally related proteins which play a fundamental role in regulating embryonic growth and differentiation as well as in maintaining homeostasis in the adults through high binding affinity for IGF-I and IGF-II [1]. In the cellular environment, IGFBP-5 can modulate IGF access to the IGF-I receptor, either inhibiting or enhancing IGF-I receptor signaling depending on the cellular context. In addition to regulating the IGF functions, the IGFBP-5 has been shown to have IGF-independent functions in regulating cell proliferation, migration and cell survival [2-4]. The molecular and cellular mechanisms of its IGF-independent functions are unknown, although increase intracellular calcium concentration [5] and interaction between IGFBP-5 and nuclear histone-DNA complex has been found [6].

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Many of the recently published studies of IGFBP-5 have focused on the involvement of IGFBP-5 nuclear trafficking [2, 7]. The C-terminal domain of IGFBP-5 contains a putative nuclear localization signal (NLS), which is responsible for the nuclear transport of IGFBP-5 [2, 8, 9]. The NLS is believed to mediate the translocation of IGFBP-5 to the nuclear compartment as IGFBP-5 was detected in the nucleus of the breast cancer cell line T47D, lung fibroblasts from patients with IPF, vascular smooth muscle cells and osteosarcoma cells [2, 10]. Understanding nuclear trafficking of IGFBP-5 may provide a means by which to intervene its nucleus function. Meanwhile active nuclear trafficking is also facilitated by the superfamily, which are further categorized into importin-α and importin-β. Mouse and human cells contain six and seven importin-α subtypes, and only one importin-β1. Acts as an adapter, importin-α binding to the NLS of cytosolic proteins, including IGFBP-5, as well as to the transport carrier, importin-β1 [11]. Research in IGFBP-5 nuclear import was analyzed and contradictory results were identified. Schedlich LJ *et al*. presumed, from the IGFBP-3 experiments, that the

mechanism by which IGFBP-5 is imported into the nucleus implies importin-β as the major transporter from the cytoplasm into the nucleus which depends on its bipartite nuclear localization sequence (NLS) [8]. While Jurgeit A *et al*. found that forced expression of a non-secreted form of IGFBP-5 results in nuclear localization, whereas upon expression of the secreted form, no nuclear localization is observed under physiological conditions and as long as the integrity of the plasma membrane remains intact, intracellular trafficking of IGFBP-5 is confined to vesicular structures in the cytoplasm and its uptake into the nucleus is impeded [12], though, the mechanism of IGFBP-5 nuclear translocation is largely unknown. Lack of sensitivity to detect low levels of nuclear IGFBP-5 could be a problem when investigating the localization of the endogenous protein.

As our previous study, we developed an unbiased luciferase report assessment for nuclear IGFBP-5 [13]. Using this assay, we have found that nucleus IGFBP-5 probably comes from ER-cytosol retro-translocation, which is a new translocation pathway in the reverse direction-called retrotranslocation-which results in the export of proteins from the lumen or membrane of the ER into the cytosol (unpublished data) [13].

In this study, our findings demonstrate that importin-α5, a nuclear import protein, was identified as a binding partner of IGFBP-5. Importin-α5 binds to IGFBP-5 form a complex with importin-β, and subsequently translocates into the nucleus. Nuclear localization sequence domain deletion eliminated nuclear localization of IGFBP-5 and significantly promotes cancer cell proliferation.

Materials and Methods

Cell culture and reagents

HeLa cells were cultured according to American Type Culture Collection guidelines. Unless otherwise noted, all chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA). Ribonuclearse-free deoxyribonuclearse and restriction endonuclearses were purchased from Promega (Madison, WI). Taq DNA polymerase and Vent DNA polymerase were purchased from New England Biolabs (Beverly, MA). Pfu Turbo DNA polymerase was purchased from Stratagene (La Jolla, CA). Superscript II reverse transcriptase (RT) and oligonucleotide primers were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). 4′-6-Diamidino-2-phenylindole (DAPI) was purchased from Sigma (St. Louis, MO). The Matchmaker^{TM} Two-Hybrid System was purchased from Clontech Laboratories (Palo Alto, CA).

Antibodies

Guinea pig polyclonal anti human IGFBP-5 was a generous gift Dr. David R. Clemmons, University of North Carolina at Chapel Hill [14]. Anti-EGFP rabbit polyclonal antibody was purchased from Torrey pines biolabs (Houston, TX) and used at a dilution of 1:5,000 for western blotting. The Anti-c-Myc mouse monoclonal antibody and anti-histone H3 rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used at a dilution of 1:3,000 for western blotting. Anti-Akt rabbit polyclonal antibody was purchased from Cell Signaling Technology (Boston, MA) and used at a dilution of 1:1,000 for western blotting. Anti-Importin-α5 mouse monoclonal antibody was bought from Novus Biologicals Inc. (Littleton, CO) and used at a dilution of 1:1,000 for western blotting. HRP-linked anti-mouse IgG and anti-rabbit IgG were bought from Jackson ImmunoResearch Laboratories (West Grove, PA) and used at a dilution of 1:10,000 for western blotting.

Yeast 2-hybrid assay

A pACT2 clone of Importin-α5 containing code for amino acids 175 to the C terminus of the protein was isolated from a human aorta cDNA library screened with IGFBP-5 lacking signal peptide as bait, as previously described [15]. Interaction was confirmed and further protein-protein interactions were assayed as described by the manufacturer where binding was determined by growth of transformed yeast colonies on -leu/-trp, and -his/-ade/-leu/-trp medium plates.

Plasmid construction

cDNA encoding EGFP, IGFBP-5 and mutants were generated by PCR and cloned into the pEGFP-N1 or pCS2+ vector to generate EGFP fusions protein. pGEX-KG-Importin-α5 (175-535) was produced by digesting the Y2H construct (pACT2) with XhoI and EcoRI to drop out the insert and then ligating it into pGEX-KG digested with the same enzymes (only for GST-Pull-down experiments). pXJ40-MYC with full length importin-α1, importin-α3, importin-α4, and importin-α7 were kindly provided by Prof. Xinmin Cao (Institute of Molecular and

Cell Biology, Singapore). pGEX-importin-α5 and pGEX-importin-β were kindly provided by Prof. Yoko Aida (Retrovirus Research Unit, RIKEN) and Prof. William W. Hall (University College Dublin, Belfield). cDNA encoding human importin-α5 and importin-β (aa 1-462) were generated by PCR from pGEX-importin-α5 and pGEX-importin-β and cloned into pcDNA.3.1/myc-His A(-). The cDNA fragment encoding full-length human IGFBP-5 was cloned into the NcoI and SalI sites of the pGEX-KG vector to produce a fusion protein with GST at the C terminus of IGFBP-5. DNA was isolated using Qiagen Plasmid mini-, maxi-, and mega- prep kits (Qiagen, Valencia, CA) and confirmed by DNA sequencing at the University of Michigan DNA Sequencing Core.

Protein expression and purification

Human recombinant Myc-His-tagged IGFBP-5 was expressed and purified as described before [16]. Briefly, IGFBP-5 was purified from conditioned supernatant of HEK 293 cells stably transfected with pcDNA3.1-IGFBP-5 plasmid. Cells were adapted to grow in DMEM serum-free medium for 2 days after they reached maximal cell density. Recombinant protein was purified utilizing nickel-charged resins (Invitrogen, Carlsbad, CA) according to the manufacturer′s instructions. To ensure the complete absence of IGF-I or -II in the preparation of IGFBP-5, affinity-purified material (100 μg) was further loaded onto a Superdex G-75 (Amersham), equilibrated, and eluted with 50% formic acid at 0.5 mL/min. At the acidity of the solvent (pH < 1), any possible bound IGF would dissociate from IGFBP and elute separately (Overgaard *et al*., 2001).

BL21 (DE3) Escherichia coli cells were transformed and grown in LB broth at 37 °C to an A600 of 0.4-0.6. Following the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.1 mM, the cells were incubated at 30 °C for 4 h. After harvesting, the cells were sonicated in Sonication buffer (50 mM Tris, pH 8.0, 0.1 M NaCl, 1 mM EDTA, 0.05% Tween 20, 1 mM EDTA, 2 μg/mL leupeptin, 4 μg/mL aprotinin, 1 μg/mL pepstatin A, and 1 mM phenylmethylsulfonyl fluoride). The cells were broken and centrifuged at 12,000g for 20 min at 4 °C, and the supernatant was collected and mixed with 1 mL of a 50% (v/v) slurry of glutathione-cross-linked agarose beads (Pharmacia, Uppsala, Sweden). The fusion protein was allowed to bind to the beads at 4 °C on a rotating wheel for 12 h. The beads with GST-IGFBP-5 and GST-importin-α5 were extensively washed with and resuspended in sonication buffer, and used for the GST pull-down assay. For the fusion protein purification, the beads with fusion proteins were freed from nucleic acid contamination using sonication buffer with 2 M NaCl, and the purified fusion protein was eluted with 100 mM Tris containing 20 mM reduced glutathione (Sigma, St. Louis, MO) [17].

The pure protein was ultrafilterated and the protein purity and integrity were routinely checked by SDS-PAGE and silver staining/Coomassie blue, the amount of purified protein was determined using BCA protein assay kit (Pierce Biotechnology, Rockford, IL), and stored at -70 °C until use.

GST pull-down

GST-IGFBP-5 and GST-importin-α5 (175-535) were purifed as described and bound to GSH-sepharose. 400 μL cell lysates or 0.8 μg pure GST-IGFBP-5 in 400 μL binding buffer 2 (20 mM NaH_2PO_4, 15 mM Tris-Ac, 0.3% Tween-20, 5 mM EDTA, 0.1% BSA) were incubated with bead-conjugated GST-fusion or GST alone overnight at 4 °C. After incubation, beads were pulled down by centrifugation and washed with biding buffer, then boiling in Laemmeli Sample Buffer. The eluted proteins were solved by SDS-PAGE and analyzed by immunoblot as described previously [18].

Co-immunoprecipitation

Co-immunoprecipitations were performed essentially by following the instructions of the manufacturers of the respective antibodies. In brief, the cells were washed three times with cold PBS and lysed in 1 mL of NP-40 buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40, and 0.25 mM EDTA [pH 7.4]) with the protease and phosphatase inhibitors. After pre-clear with 20 μL protein G for 1 h at 4 °C, cell lysate was incubated with the appropriate antibody (2 μg) and protein G (20 μL) over night at 4 °C. The immunoprecipitates were washed once with NP-40 buffer and three times with PBS with protease and phosphatase inhibitors, and then separated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (PVDF), followed by immunoblot analysis with appropriate antibodies.

Transcription assay

Hela cells were plated in 6 wells and grown for 24 h. Transfections were performed using Lipofectamine

2,000 (Invitrogen, Carlsbad, CA) with 100 ng Renilla luciferase, 400 ng Gal4-luc, and 500 ng of ERX plasmid and 500 ng of importin-α5/importin-β (1-462)/pcDNA3.1 as indicated. Transfections were performed in duplicate, measured using the Dual Luciferase System (Promega, Madison, WI) 24 h post-transfection.

RNA interference (RNAi)

siGENOME SMARTpool short interfering RNA (siRNA) duplexes specific for human importin-α5 were prepared (Dharmacon Research, Layfayette, CO) and transfected with Lipofectamine 2,000 (Invitrogen, Carlsbad, CA). Forty-eight hours after siRNA transfection, cells were transfected with IGFBP-5-EGFPs and processed 24 h later.

RT-PCR

RNA extraction was performed with TRIzol (Invitrogen, Carlsbad, CA) and cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). PCRs were performed by using the following primers at their optimal condition: importin-α3, forward 5′-ATGCTTCAAGTGATAACCAAGG and reverse 5′-CAAGACAATGGACTAAAATGG; importin-α5, forward 5′-TCGCCTGAAAAGTTACAAGAA and reverse 5′-AGAAGTGATGACACCACCTGG; importin-α7, forward 5′-GTCTCACTGGGCTGCATCAAAC and reverse 5′-CTGCTGGAAGATGAACTGCTG; and β-actin, forward 5′-GAGCTACGAGCTGCCTGACG and reverse 5′-GTAGTTTCGTGGATGCCACAG.

Immunofluorescence

Cells grown on coverslips were transfected with various expression plasmids. 24 h later, cells were fixation in 4% paraformaldehyde for 1 h at room temperature. After three time wash with Milli-Q water, cells were stained with DAPI (50 ng/mL) for 15 min. Cells were then washed three times and mounted with Mounting Medium (Southern Biotech, Birmingham, Al), and examined using confocal microscope (Leica, Wetzlar, Germany). The images were processed with Adobe Photoshop software (Adobe, San Jose, CA).

Subcellular fractionation

For preparation of the cytosol and nuclear fraction, 1×10^7 cells were required using the nuclear/cytosol fractionation kit in accordance with the manufacturer′s protocol (BioVision, Inc., Mountain View, CA).

Binding studies using surface plasmon resonance assay

To characterize the binding of IGFBP-5 with importin-α5 or importin-β, binding assay, kinetics were examined using surface plasmon resonance (SPR) assays (BIAcore, Amersham Pharmacia, Uppsala, Sweden). The IGFBP-5 sensor chip was prepared according to the manufactory′s manual. All binding experiments were performed at 25 °C with a constant flow rate of 5 μL/min HBS-EP buffer (HBS-EP buffer: 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20 (v/v), pH 7.4), except for the kinetics assay, which utilized a constant flow rate of 15 μL/min HBS-EP. To correct for non-specific binding and the bulk refractive index change, a blank channel (FC2) without importin-α5/importin-β was employed as a control for each experiment. Sensorgrams for all binding interactions were recorded in real time and analyzed after subtracting that from the blank channel. Changes in mass due to the binding response were recorded as response units (RU). Between experiments, the sensor chip surface was regenerated with 60 μL of 2 M NaCl [17].

Statisitics

Statistical significance among each experimental group was determined by one-way analysis of variance (ANOVA), followed by the Newman-Keuls maltiple comparison test. All values are represented as means ± standard error (S.E.). Calculation was performed using GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA), and significance was accepted at $p<0.05$.

Results

Identification of importin-α5 as an IGFBP-5-interacting Protein

In order to isolate possible binding partners for IGFBP-5, a construct of the mature protein was fused to yeast GAL4 activation domain. This construct was used as "bait" to screen a human aorta cDNA library. Of several million clones screened, 63 were identified that bound IGFBP-5 under high stringency (3x). Importin-α5 (4 of 63 clones coded an importin-α5 fragment from amino acid 175 to the end) was selected for further examination because of our interest in transport of IGFBP-5 to the nucleus. While this 57 kD protein has been suggested as a cytoplasmic chaperone to guard against aggregation of nuclear localization sequence, its well-studied role is

as an adaptor molecule in importin-α/β mediated nuclear import [11, 15, 19].

In order to further investigate importin-α5/IGFBP-5 binding, yeast two hybrid assays were performed using the isolated importin-α5 fragment (aa 175 to the end) (n=10) (Fig. 1A). All of the yeast transformants grew on leucine- and tryptophan-deficient plates, but not plasmids transfected group, indicating successful transformation (Fig. 1A, left panel). When plated on the selective plates (leucine-, tryptophan-, histidine-, adenine- deficient), only the cells co-transformed with both IGFBP-5 and importin-α5 were able to grow (Fig. 1 A, right panel b′).

The protein-protein interaction was then shown *in vitro* by fusing GST upstream of IGFBP-5. The purified fusion protein immobilized beads successfully pulled endogenous importin-α5 out of pVSMC cell lysates while GST alone did not (Fig. 1B, upper panel). GST-importin-α5 (aa 175 to the end) beads were produced and allowed to bind in solution to native IGFBP-5. Binding was detected by immunoblot and was undetectable when GST was substituted for GST-importin-α5 (175-535) (Fig. 1B, lower panel).

To test whether IGFBP-5 and importin-α5 interact with each other under physiological conditions, co-immunoprecipitation experiments were performed using Hela cells. We transfected EGFP-tagged IGFBP-5, or the EGFP plasmid, to the HeLa cells and measured their interaction with the endogenous importin-α5 proteins. The cell lysates were immunoprecipitated (IP)

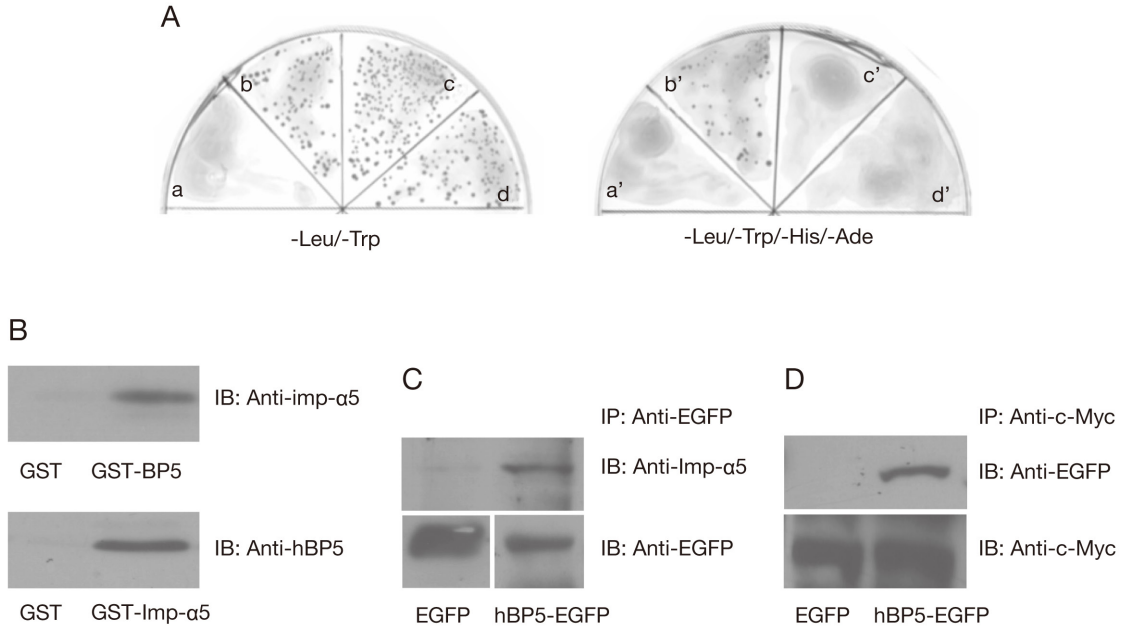

Fig. 1 Identification of importin-α5 as an IGFBP-5-interacting protein

A, IGFBP-5 interacts with importin-α5 in the yeasts. Yeasts were transformed with no DNA (a, a'), pGBKT7-IGFBP-5/pGADT7-Importin-α5 (b, b'), pGBKT7-IGFBP-5/pGADT7 (c, c'), pGBKT7/pGADT7-importin-α5 (d, d'). The yeast transformants were grown on leucine- and -tryptophan-deficient plates (left panel) or the leucine-, tryptophan-, adenine-, and histidine- deficient plates (right panel). **B**, *in vitro* interaction between importin-α5 and IGFBP-5. Hela cells lysates were incubated with GST or GST-IGFBP-5/GST-importin-α5 immobilized on glutathione-Sepharose beads at 4 °C for 3 h. The pulled-down complex was analyzed by western blot analysis using anti-IGFBP-5 or anti-importin α5 antibody. **C**, Interaction of the endogenous importin-α5 with transfected IGFBP-5. IGFBP-5-EGFP or EGFP alone was transiently expressed in HeLa cells. 24h later, the cells were lysis with NP-40 buffer and immunoprecipitated with anti-EGFP (2 μg) antibody, and subjected to western blot analysis to detect bound importin-α5 (upper panel). IP efficiency is shown in lower panel. **D**, Interaction of the transfected IGFBP-5 and importin-α5. Hela cells were co-transfected with IGFBP-5-EGFP/EGFP and importin-α5 together. 24h later, the cells were lysis with NP-40 buffer and immunoprecipitated with anti-c-Myc (2 μg) antibody, and subjected to western blot analysis to detect bound IGFBP-5 (upper panel). IP efficiency is shown in lower panel. Results are representative of three independent experiments.

with anti-EGFP antibody, and subjected to western blot analysis to detect the bound importin-α5 proteins with anti-importin-α5 antibody (Novus Biologicals Inc, Littleton, CO) (Fig. 1C, upper panel). The association of IGFBP-5 and importin-α5 was also confirmed in a reciprocal co-immunoprecipitation experiment. Hela cells were co-transfect with c-Myc tagged importin-α5 and IGFBP-5-EGFP/EGFP. Cell lysates were immunoprecipitated with anti-c-Myc antibody and immunoblot with anti-EGFP antibody. C-Myc tagged importin-α5 co-immunoprecipitated with IGFBP-5-EGFP, but not EGFP (Fig. 1D, upper panel). Also, the IP efficiency and expression levels of various importin-α5 and EGFP/IGFBP-5-EGFP were comparable (Fig. 1C, D lower panel). The results showed that IGFBP-5 was able to bind cellular importin-α5 both in biochemical conditions and in physiological conditions.

Binding affinity of IGFBP-5 and importins were determined by surface plasmon resonance technology, an optical technique utilized for quantitative analyses of binding affinity in molecular interactions. The amount of importin-α5/importin-β bound to a constant amount of IGFBP-5 in the sensor tip was measured as resonance units at five different concentrations of importins. Importin-α5/importin-β did bind to IGFBP-5, and the amount of bound importins increased with rising importins concentrations with a dissociation constant K_D=3.4e-7 (IGFBP-5/importin-α5) and K_D=2.44e-7 (IGFBP-5/importin-β), respectively (Table 1).

Nuclear import of IGFBP-5 is mediated by the importin-α5/β pathway

iGFBP-5 subcellular localization determination using confocal microscopy and cell fractionation

The classic nuclear protein import is binding of the import substrate (nuclear proteins) to the importin-α/β heterodimer; α subunit provides the binding site for the NLS of the import substrate. The resulting trimeric complex then docks to the cytoplasmic periphery of the nuclear pore complexes (NPC), and is subsequently translocated to the nuclear side of the NPC [8, 11, 19]. To determine whether the interaction between importin-α5 and IGFBP-5 is result to the IGFBP-5 nuclear import, we investigated the subcellular localization of EGFP-tagged IGFBP-5 with (hIGFBP-5-EGFP) or without (mIGFBP-5-EGFP) signal peptide by confocal microscopy (Fig. 2A and 2B). Deletion signal peptide of IGFBP-5 resulted more nuclear deposition compare with wild type IGFBP-5 (Fig. 2B a and a′). Blockage of importin pathway by knockdown importin-α5 (Fig. 2B c and c′) or by competing functional importin-β using dominant negative importin-β mutant (Fig. 2B d and d′), both significantly increased nuclear EGFP signal in the cytoplasmic. While overexpress of importin-α5 did not show significant alteration of nuclear/cytoplasmic EGFP signal (Fig. 2B b and b′).

To confirm the subcellular localization described above, we fractionated the corresponding cell lysates and assayed for the amount of proteins contained in the nuclear *versus* the cytoplasmic fractions for each group by western blotting. The nuclear fraction was identified by the presence of the nuclear protein, histone H3, whereas the ex-nuclear fraction was confirmed by Akt immunoblot (Fig. 3).

Firstly, we examined changes in the levels of cytoplasmic and nuclear IGFBP-5 after overexpression of the importin-α5. After co-transfect with importin-α5 and hIGFBP-5-EGFP/mIGFBP-5-EGFP, most EGFP signals were co-localized in the nucleus, while the EGFP signals were hardly seen in the cytoplasmic (Fig. 2B b and b′). Cell fractionation results showed that most hIGFBP-5-EGFP (Fig. 3A) and mIGFBP-5-EGFP (Fig. 3B) are in the nuclear fraction in control group, while a little is in the cytoplasmic fraction. After overexpressed importin-α5, there is no significant changes have been found on ratio of nucleus fraction and cytoplasmic fraction between importin-α5 overexpress group and control group (Fig. 3A and B).

To further test the hypothesis that importin-α5 is a carrier of hIGFBP-5 into the nucleus, we used RNAi to inhibit the expression of importin-α5 and evaluated the effect on the localization of hIGFBP-5-EGFP. After cultures were treated with siRNA for 48 h, either hIGFBP-5-EGFP or mIGFBP-5-EGFP expression plasmid was transfected and cellular localization was evaluated. It was

Table 1 Kinetic parameters of interaction between of IGFBP-5 and importin-α5/importin-β

	k_a (1/Ms)	k_d (1/ s)	K_A (1/M)	K_D (M)
Importin β	1.9e4	4.65e-3	4.09e6	2.44e-7
Importin α5	2.91e3	9.92e-4	2.94e6	3.4e-7

ka, association rate constant; kDa, dissociation rate constant; KA, equilibrium association constant; KD, equilibrium dissociation constant. Data shown are three independent experiments with similar results.

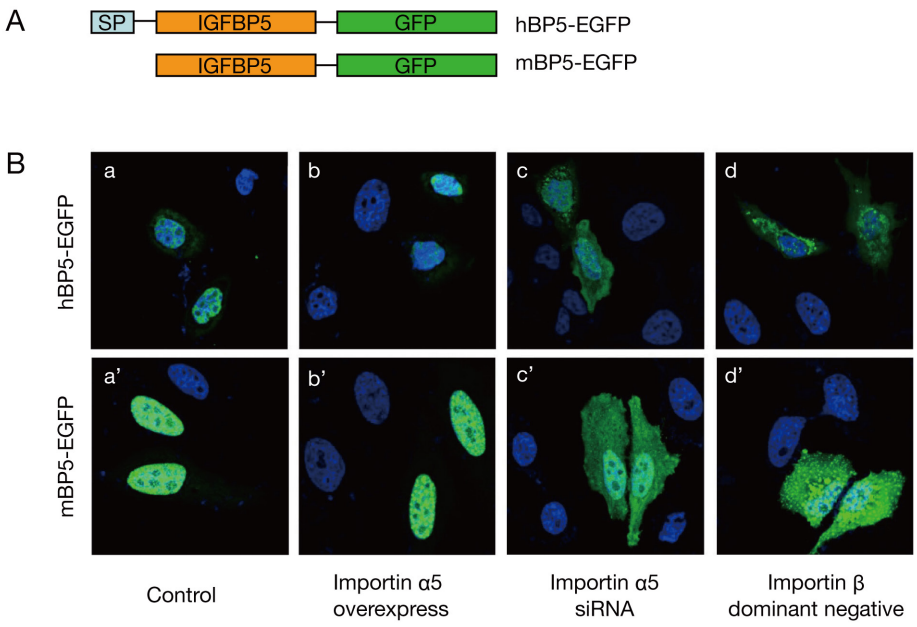

Fig. 2 Subcellular localization of IGFBP-5

A, Diagram of constructs used to study subcellular localization of IGFBP-5-EGFP. **B**, Subcellular localization of transfected IGFBP-5s. Cellular localization was determined by confocal immunofluorescence microscopy and is shown as an overlay of DAPI (nuclear) and EGFP signal (IGFBP-5s). Hela cells were co-transfected with hIGFBP-5-EGFP (left panel)/mIGFBP-5-EGFP (right panel) plasmid with pcDNA3.1 (a, a'), and importin-α5 (b, b'), and importin-β (1-462) (d, d'). For the siRNA group (c, c'), cells were transfected with siRNA 48 h before transfected with IGFBP-5s. 24 h after transfection with IGFBP-5s, cells were extensively washed, fixed, and the nuclei were determined by DAPI. Then, cellular fluorescence was visualized by confocal microscopy. Results are representative of three independent experiments.

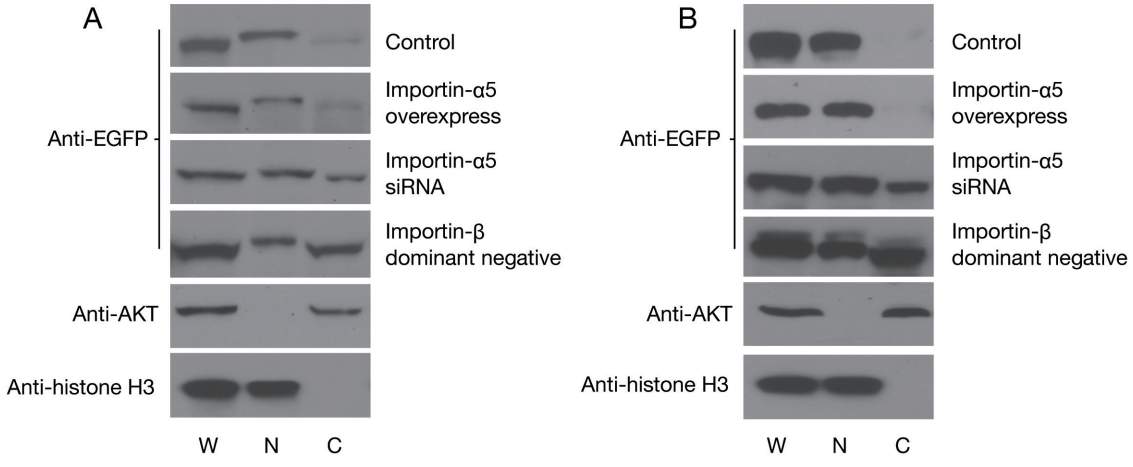

Fig. 3 Subcellular distribution of IGFBP-5

Hela cells were transfected with hIGFBP-5-EGFP (**A**)/mIGFBP-5-EGFP (**B**) plasmid with pcDNA3.1 (control)/importin-α5/importin-β (1-462) as indicated. For the siRNA group, cells were transfected with siRNA 48 h before transfected with IGFBP-5. 24 h after transfection with IGFBP-5s, cells were fractionated into whole cell lysate (W), nuclear fraction (N), and cytosolic (C), using nuclear/cytosol fractionation kit. The fractions were subjected to western blotting analysis using anti-EGFP antibody. And, the same fractions were also analyzed using antibodies against Akt, and histone-H3 to confirm the different fractions. Results are representative of three independent experiments.

readily apparent that cultures transfected with importin-α5 siRNA showed a distinct subcellular localization of control IGFBP-5-EGFPs with obviously increased cytoplasmic signaling and decreased nucleus signaling (Fig. 2B c and c′). As shown in cell fractionation results (Fig. 3A and B), most of hIGFBP-5-EGFP and mIGFBP-5-EGFP were detected in the nuclear fraction at control group. When importin-α5 expression was silenced after infection with synthesis siRNA, both cytoplasmic hIGFBP-5-EGFP and mIGFBP-5-EGFP were increased. In contrast, the relative amounts of protein detected in the nucleus in RNA silence group were reduced as compared with control group.

As we know, dominant-negative mutants of importin-β block classic nuclear import pathways mediated by importin-α/β complex. To confirm our hypothesis that IGFBP-5 is carried by the importin-α/β complex into the nucleus, importin-β dominant-negative mutant was introduced to block the nuclear import pathway, and the effect on the cellular localization of IGFBP-5-EGFP was evaluated. Importin-β dominant-negative plasmid and hIGFBP-5-EGFP/mIGFBP-5-EGFP plasmid were co-transfected to Hela cells. 48 h after transfection, cells were visualized using confocal microscopy. EGFP signal was predominantly localized in the cytoplasmic space (Fig. 2B d and d′) in the transfected cells with importin-β dominant-negative mutant and IGFBP-5-EGFPs plasmids. In addition, cell nucleus and cytoplasmic were separated using nuclear/cytosol fractionation kit, and analyzed by western blotting. Western blotting results confirmed the confocal microscopy observation. After blocked importin-α/β nuclear import pathway with importin-β dominant-negative mutant, cytoplasmic IGFBP-5-EGFPs level are comparable with them in the nuclear, while in control group, nuclear IGFBP-5-EGFPs level are much higher as compared to them in the cytoplasmic (Fig. 3A and B).

Quantify IGFBP-5 nuclear translocation using luciferase report assay

To quantify the effect of importin-α/β pathway on IGFBP-5 nuclear translocation, we therefore established a quantitative system to measure the nuclear IGFBP-5 based on the calreticulin retrotranslocation assay [13, 20]. IGFBP-5 cDNA was tagged with ERX, which composed of the Gal4 DNA binding domain and the p53 activation domain. ERX-tagged IGFBP-5 with (hIGFBP-5-ERX) or without (mIGFBP-5-ERX) signal peptide were also been demonstrated separately (Fig. 4A). After co-transfected with ERX plasmids and luciferase report plasmid, IGFBP-5-ERX localized in the nuclear is able to activate the Gal4 dependent luciferase and the amount of nuclear IGFBP-5-ERXs are correlated with the luciferase activity. In Hela cells, hIGFBP-5-ERX and mIGFBP-5-ERX showed 35.15±1.39 and 87.00±1.86 folds of nuclear activity compared to baseline control respectively (Fig. 4B). These data are consistent with our observation that mIGFBP-5-ERX shows much stronger nuclear localization than hIGFBP-5-ERX in Hela cells (Fig. 2B, a and a′).

After co-transfected IGFBP-5-ERX and importin-α5, it was readily apparent that the transcriptional activity IGFBP-5-ERX did change significantly as compared with the group with importin-α5 transfection (Fig. 4C). While, after treated the cell with importin-α5 siRNA, as shown in Fig. 4D, the transcriptional activity IGFBP-5-ERX dropped down to 53% (hIGFBP-5-ERX) and 33% (mIGFBP-5-ERX), respectively, as compared to control siRNA group. The specificity and the efficiency of siRNA were examined by western blotting (Fig. 4F) and RT-PCR (Fig. 4G). As shown in Fig. 4E, importin-β dominant negative showed obvious effect on blocking importin-α/β nuclear import pathway. Co-transfected importin-β dominant negative plasmid and hIGFBP-5-ERX/mIGFBP-5-ERX plasmid dramatically decreased hIGFBP-5-ERX transcriptional activity from 28.27±5.74 to 6.51±0.12, and mIGFBP-5-ERX transcriptional activity from 71.51±1.60 to 7.59±0.63. These results indicate that IGFBP-5 nuclear import through importin-α/β nuclear import pathway.

Nuclear import of IGFBP-5 requires a functional NLS in IGFBP-5 C-domain

To address the possibility that the hypothetic NLS sequence of IGFBP-5 is functionally and is involved in IGFBP-5 nuclear import, interaction between IGFBP-5 NLS mutant and importin-α5 was tested using yeast two hybrid first. When the IGFBP-5 without NLS domain mutant plasmids were introduced into cells together with importin-α5 plasmid, cells was able to grow on leucine- and tryptophan- deficient plates (Fig. 5A left panel), but not the leucine-, tryptophan-, histidine-, and adenine- deficient plates (Fig. 5A right panel). The result suggests that NLS of IGFBP-5 is required for its binding interaction with importin-α5.

To study whether the interaction between importin-α5 and IGFBP-5 is in important in IGFBP-5 nuclear localization, we generated a mutant of

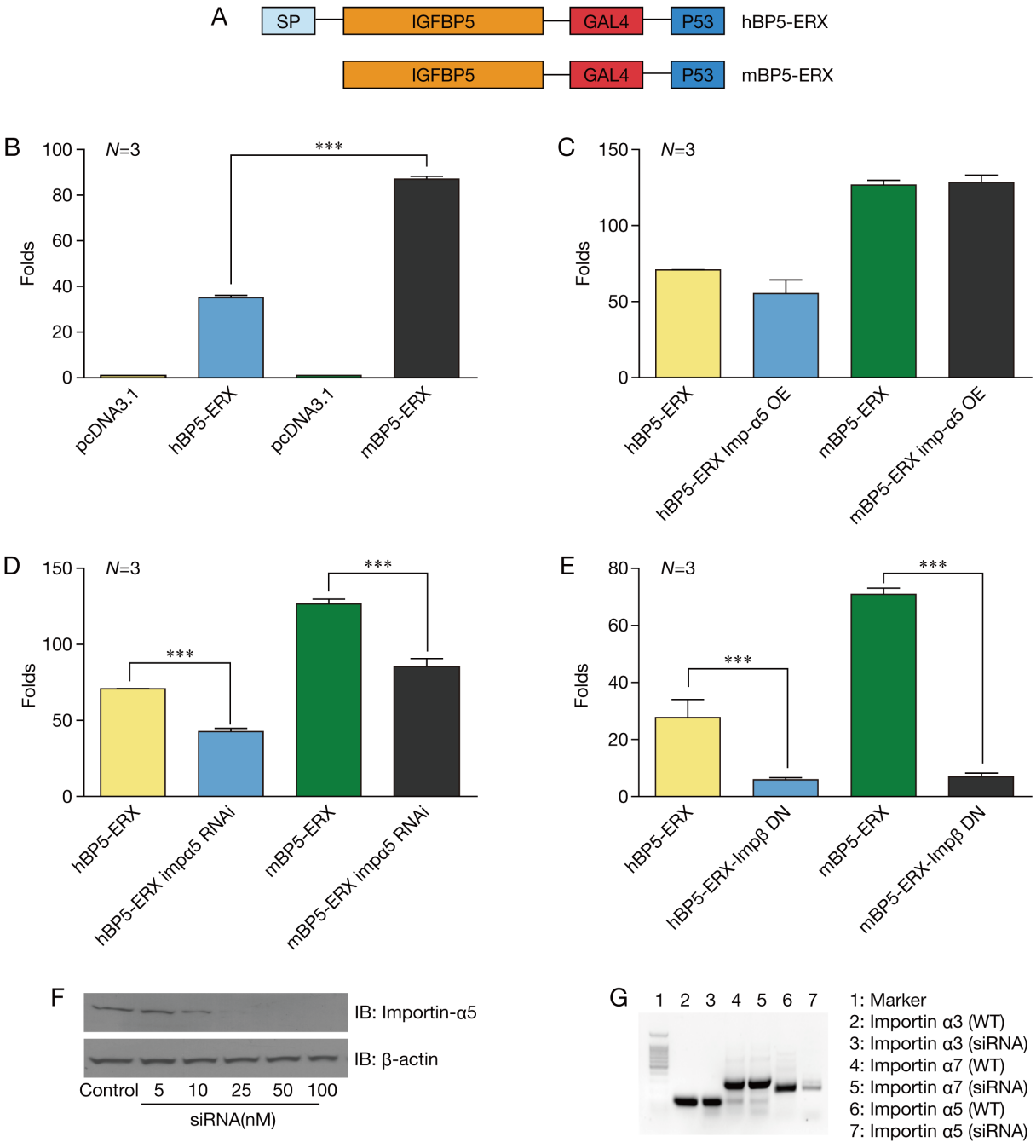

Fig. 4 Transcription-based assay to measure nuclear IGFBP-5

Transcriptional activity was determined and the transcriptional repressor activity is expressed as -fold decrease over the pBind control group. **A**, Diagram of constructs used for study transcription-based assay. **B**, **C**, **D** and **E** are transcription-based assay. **B**, Hela cells were transfected with ERX plasmids or pcDNA3.1. **C**, Hela cells were transfected with ERX plasmids/ pcDNA3.1 with importin-α5. **D**, Hela cells were transfected with ERX/pcDNA3.1 after 48 h importin-α5 siRNA transfection. **E**, Hela cells were transfected with ERX plasmid/pcDNA3.1 with dominant negative importin-β (1-462). 24 h after transfected with plasmids as indicated (B, C, D, E), cell lysates were measured using the dual luciferase system and the luciferase assays were normalized to co-transfected renilla luciferase, and are plotted with the standard deviation. *** $p<0.001$. **F**, Reduction of importin-α5 protein level were detected by western blot, β-actin was using as internal control. **G**, Reduction of mRNAs encoding importin α3, importin-α5 or importin-α7 proteins were detected by RT-PCR using specific primers after 48 h transfection. Results are representative of three independent experiments.

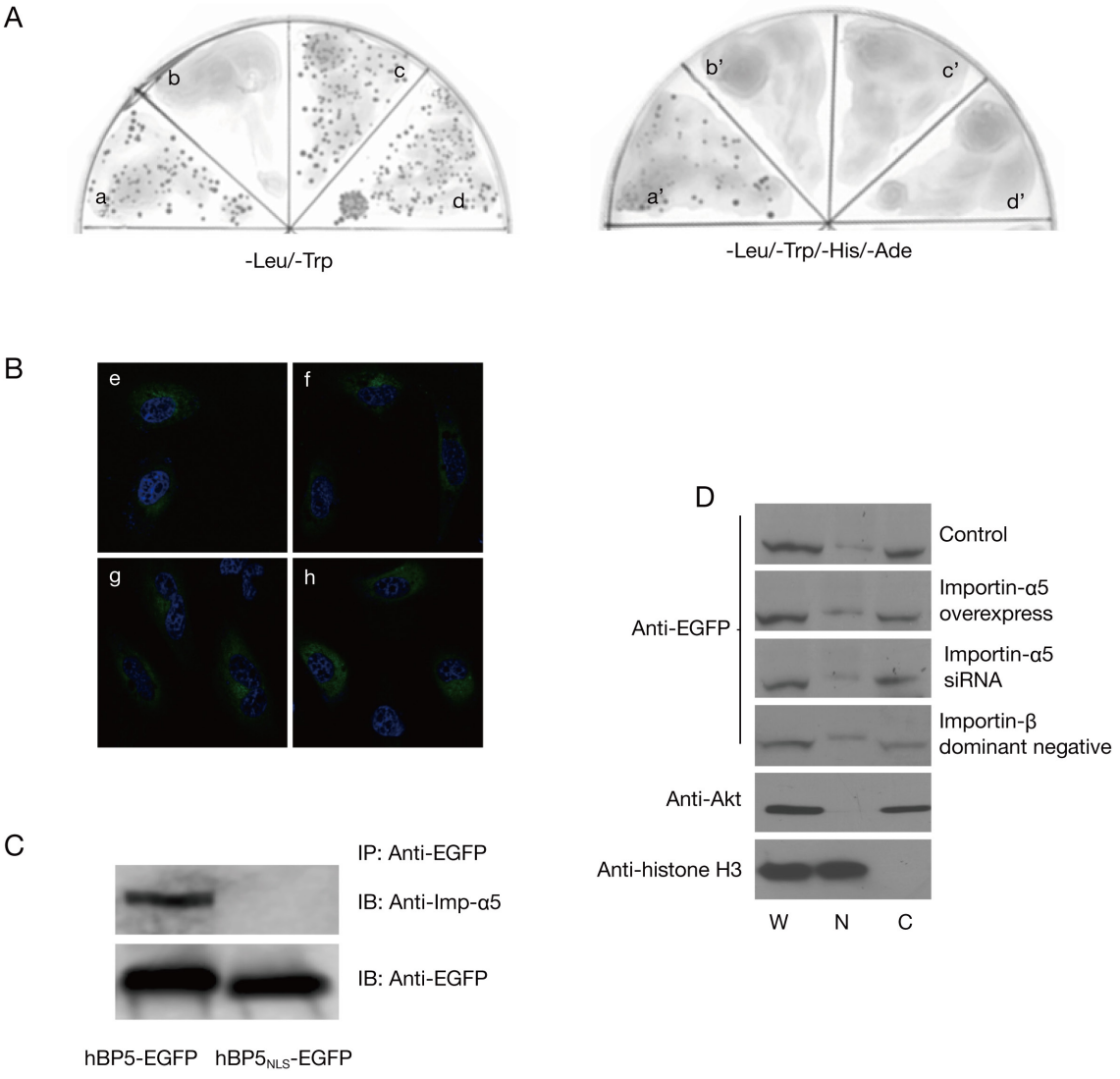

Fig. 5 IGFBP-5 contains a functional nuclear localization signal

A, IGFBP-5 NLS is required interact with importin- α5. Yeasts were transformed with pGBKT7-IGFBP-5/pGADT7-Importin-α5 (a, a'), pGBKT7-IGFBP-5_{NLS}/pGADT7-Importin-α5 (b, b'), pGBKT7-IGFBP-5/pGADT7 (c, c'), pGBKT7/pGADT7-importin-α5 (d, d'). The yeast transformants were grown on leucine- and -tryptophan-deficient plates (left panel) or the leucine-, tryptophan-, adenine-, and histidine- deficient plates (right panel). **B**, Subcellular localization of transfected IGFBP-5 without NLS. Cellular localization was determined by confocal immunofluorescence microscopy and is shown as an overlay of DAPI (nuclear) and EGFP signal (IGFBP-5_{NLS}). Hela cells were co-transfected with IGFBP-5_{NLS} plasmid with pcDNA3.1 (e), and importin-α5 (f), and importin-β(1-462) (h). For the siRNA group (g), cells were transfected with siRNA 48 h before transfected with IGFBP-5_{NLS}. 24 h after transfection with IGFBP-5_{NLS}, cells were extensively washed, fixed, and the nuclei were determined by DAPI. Then, cellular fluorescence was visualized by confocal microscopy. **C**, No interaction between IGFBP-5_{NLS} and importin-α5. Hela cells were co-transfected with IGFBP-5-EGFP/ IGFBP-5_{NLS}-EGFP and importin-α5 together. 24h later, the cells were lysis with NP-40 buffer and immunoprecipitated with anti-EGFP (2 μg) antibody, and subjected to western blot analysis to detect bound importin-α5 (upper panel). IP efficiency is shown in lower panel. **D**, Subcellular distribution of IGFBP-5. Hela cells were transfected without NLS plasmid with pcDNA3.1 (control)/ importin-α5/importin-β(1-462) as indicated. For the siRNA group, cells were transfected with siRNA 48 h before transfected with IGFBP-5_{NLS}. 24 h after transfection with IGFBP-5_{NLS}, cells were fractionated into whole cell lysate (W), nuclear fraction (N), and cytosolic (C) using nuclear/cytosol fractionation kit. The fractions were subjected to Western blotting analysis using anti-EGFP antibody. And, the same fractions were also analyzed using antibodies against Akt, and histone-H3 to confirm the different fractions. Results are representative of three independent experiments.

IGFBP-5 that contained a deletion of residues 201-218 that spans the predicted NLS. The subcellular localization of IGFBP-5_{NLS}-EGFP was monitored using confocal microscopy. As shown in Fig. 5B (e), the IGFBP-5_{NLS}-EGFP was localized to the cytoplasm, being generally excluded from the nucleus. When overexpress importin-α5, NLS-deleted mutant was also detected only in the cytoplasm of transfected cells implying that IGFBP-5_{NLS} does not interact with importin-α5 in cultured cells. SiRNA of importin-α5 and importin-β dominant negative were also introduced to study the effect of importin-α/β nuclear import pathway on IGFBP-5 NLS mutant subcellular localization. As shown in Fig. 5B (g and h), IGFBP-5 NLS mutants were found to locate primarily in the cytosol, and no EGFP signal co-localized with nuclear maker, DAPI. We also tested IGFBP-5 NLS mutant interaction with importin-α5 by immunoprecipitation. Our result (Fig. 5C) showed that anti-EGFP antibody did not co-immunoprecipitated IGFBP-5 without NLS sequence in cultured cells. We believe that NLS is critical on IGFBP-5 nuclear transport. To confirm the mutant phenotypes described above, we fractionated the corresponding cell lysates and assayed for the amount of proteins contained in the nuclear *versus* the cytoplasmic fractions by western blotting. As shown in Fig. 5D, a significant amount of mutant IGFBP-5 was detected in the cytoplasmic fraction, while little protein was detected in the nuclear fraction. Overexpress importin-α5, siRNA importin-α5, importin-β dominant negative blocking importin-α/β pathway do not change the relative amounts of protein detected in the nucleus and cytoplasmic fractions. Confocal microscopy (Fig. 5B), co-immunoprecipitation (Fig. 5C), and western blotting (Fig. 5D) results demonstrated that the fragment spanning residues 201-218 is the functional NLS for IGFBP-5. It is required for binding with importin-α5 and IGFBP-5 nuclear import.

Discussion

In this study we have analyzed the nuclear import pathways of the insulin-like growth factor binding protein-5 in detail. The classic import receptor, the importin-β, had previously been postulated as a nuclear import partner for IGFBP-5 instead of importin-α [8]. The authors of this study, however, suggested that importin-α5 serves as an initial binding partner for IGFBP-5. Our results now clearly show that importin-α5 indeed binds to IGFBP-5 with moderate affinity, and the importin-α5/β complex serves as adapter together involved in importing IGFBP-5 into the nucleus.

Besides IGF dependent effect, IGFBP-5 has also been documented ligand-independent effects of on cell growth, migration, and apoptosis/survival in a number of cell types [2, 4]. Our previous studies showed that IGFBP-5 is a protein has two destinations, extracellular or nucleus [13]. The endogenous IGFBP-5 is found in the nuclei of vascular smooth muscle cell culture and mouse embryonic cartilage cells and the N-domain of IGFBP-5 possess transactivation activity when fused to the DNA-binding domain of Gal4. FHL2 (four-and-a-half LIM domain 2), a transcriptional regulator, was found to interact with IGFBP-5 [21, 22]. And also IGFBP-5 interacts with nuclear vitamin D receptor (VDR) to prevent retinoid X receptor (RXR): VDR heterodimerization [23]. Lots of studies implied that IGFBP-5 may interact with transcriptional regulators to exert its IGF-independent functions [4]. The mechanism(s) for the IGF-independent effects of IGFBP-5 are currently unknown but may involve a direct nuclear action. Previous reports postulated that importin-β dependent nuclear uptake is involved in IGFBP-5 nuclear import, based on the IGFBP-3 results [9]. Herein, we use yeast two hybrid to screen IGFBP-5 binding partner and find that importin-α5 interacts with IGFBP-5. Given that importin-α family is one of the component of the best understood system for the transport of macromolecules between the cytoplasm and the nucleus [11, 24]. Defined by the primary amino acid similarities, Importin-αs are classified into three subfamilies in human (clade 1 to 3), and importin-α5 is referred to clade α1 with importin-α6, and α7. Importin-α5 have been demonstrated that mediated the nuclear translocation of some protein, like NF-κB, STAT3 *etc*, and the expression of importin-α5 in human cells could be regulated by miR233 [11]. GST pull-down and co-IP were carried out to confirm the interaction between importin-α5 and IGFBP-5. Besides, overexpress and knockdown of importin-α/β pathway were introduced to demonstrate the hypothesis, importin-α5 and importin β are both required for IGFBP-5 nuclear translocation, rather than a particular importin only. The individual cells were visualized using confocal microscopy and subcellular localization of EGFP-tagged IGFBP-5

has been carried out based on IGFBP-5 localization. Moreover, luciferase report assay quantified the nuclear IGFBP-5 for the entire cell population in variety conditions.

As shown in the confocal microscopy (Fig. 2B), cell fractionation (Fig. 3), and report assay results (Fig. 4C), increase of importin-α5 failed to enhance IGFBP-5 nuclear import. While, in the control group, we found that the basal efficiency of IGFBP-5 nuclear import is quite high, and the cytoplasmic IGFBP-5 is hardly seen in Fig. 2B (a and a′). That IGFBP-5 nuclear cytoplasmic distribution pattern implicated that endogenous importin-α5 is sufficient to bind to the IGFBP-5, mediate the nuclear import of cytoplasmic IGFBP-5. That might explain that further increase of importin-α5 had no enhancement on IGFBP-5 nuclear import.

RNAi and importin-β dominant negative mutant was also used to corroborate the function of importin-α/β complex in IGFBP-5 nuclear import from report assay. Transient transfection of importin-α5 siRNA has limited effect on blocking IGFBP-5 nuclear import (about 45%), while the importin-β dominant negative mutant produced dramatic effects on inhibiting IGFBP-5 nuclear import (higher than 75%). This is probably due to compensation by other importin-αs when we knockdown importin-α5, since there are five ubiquitously expressed importin-αs. While there is only one importin-β, so no compensation has been found when we inhibited importin-α/β pathway with importin-β dominant negative mutant [11, 24].

There is one report in 2007 that IGFBP-5 enters vesicular structures but not the nucleus. Jurgeit A *et al*. did not detect endogenous IGFBP-5 in the nuclear and he speculated that they observation of nuclear uptake of IGFBP-5 was restricted to artificial conditions such as expression of non-secreted forms of IGFBP-5 or selective permeabilization of the plasma membrane by digitonin [14]. In our experiments, we transfected full-length IGFBP-5 tagged ERX, without permeabilized the cells, and found that the nuclear IGFBP-5 retrotranslocated from ER and also secreted form, which Jurgeit A *et al*. supposed so. Those observations of Jurgeit A were probably due to low endogenous expression levels or the sensitivity of the antibody. But the exogenous IGFBP-5 does not enter into the nuclear which is consistent in both studies.

In classic importin-α/β nuclear import pathway, a protein containing a classical basic nuclear localization signal (NLS) is imported by a heterodimeric import receptor consisting of importin-β, which mediates interactions with the nuclear pore complex, and the adaptor protein importin alpha, which directly binds the classical NLS. The first step of nuclear import occurs when an importin discriminates between its cargo and other cellular proteins. Proteins destined for transport into the nucleus contain amino acid targeting sequences called nuclear localization signals (NLSs). The best characterized transport signal is the classical NLS for nuclear protein import, which consists of either one (monopartite) or two (bipartite) stretches of basic amino acids. Here, we report that a bipartite stretches of basic amino acid residues in the C-terminal domain of the IGFBP-5, could function as its NLS, in the context of IGF- independent nuclear action of IGFBP-5. IGFBP-5 mutated the predicted NLS lost the binding activity with importin-α5. Deletion of predicted NLS resulted in IGFBP-5 cytoplasmic storage. The identification of NLS could also demonstrate IGFBP-5 nuclear import through importin-α/β complex in the other side.

In summary, out results demonstrated that IGFBP-5 protein is able to be imported into the nucleus by importin-α/β complex-dependent transport. IGFBP-5 nuclear localization is dependent on the NLS domain involved in the interaction with importin-α5 proteins leading to nuclear import of IGFBP-5, which should support approaches to interfere with its function in cell in an IGF-independent way. And there is one interesting observation that punctate staining pattern has been visualized in mIGFBP-5-EGFP transfected cells. The punctate staining pattern of the IGFBP-5 in the nuclear remains to be characterized but may indicate that importin-α5 plays a role in the nuclear.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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