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USP15 attenuates IGF-I signaling by antagonizing Nedd4-induced IRS-2 ubiquitination

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ABSTRACT

Insulin receptor substrates (IRSs) are phosphorylated by IGF-I receptor tyrosine kinase in a ligand-dependent manner. In turn, they bind to and activate effector proteins such as PI3K, leading to various cell responses including cell proliferation. We had reported that ubiquitin ligase Nedd4 induces mono-ubiquitination of IRS-2, thereby enhancing IRS-2 tyrosine phosphorylation, leading to increased IGF signaling and mitogenic activity. Here we show that ubiquitin-specific protease 15 (USP15) antagonizes the effect of Nedd4 on IRS-2. We identified USP15 as a protein that preferentially bound to IRS-2 when IRS-2 was conjugated with ubiquitin. In HEK293 cells, Nedd4 overexpression induced IRS-2 ubiquitination, which was decreased by USP15 co-expression while increased by USP15 knockdown. Nedd4 overexpression enhanced IGF-I-dependent IRS-2 tyrosine phosphorylation, and USP15 co-expression suppressed it. Conversely, USP15 knockdown increased IRS-2 tyrosine phosphorylation and downstream signaling in prostate cancer PC-3 cells. We concluded that USP15 attenuates IGF-I signaling by antagonizing Nedd4-induced IRS-2 ubiquitination.

1. Introduction

Insulin-like growth factors (IGFs) are peptide hormones similar in molecular structure to insulin. IGFs promote proliferation, differentiation, and survival of various cell types, and maintain diverse cellular functions [1]. IGFs play a fundamental role in body growth control. IGF-I is synthesized in the liver and other peripheral tissues under the control of growth hormone (GH), and mediates the growth promoting effect of GH [2]. IGF-I action is modulated by nutritional factors, resulting in adaptive growth to nutritional conditions [3]. In addition, local IGF-I action is often potentiated by other local hormones, causing tissue specific expression of IGF-I activity [4]. On the other hand, accumulated reports show excess IGF production and/or IGF signaling in many types of cancers, contributing to cancer development [5].

Insulin receptor substrates (IRS)-1/2 are important mediators of IGF/insulin signaling, and their tyrosine phosphorylation is a key reaction in the signal transduction. When IGFs/insulin bind to their specific receptors, the intrinsic tyrosine kinases are activated and phosphorylate IRSs. Phosphorylated IRSs are then recognized by Src homology 2 (SH2) domains in effector proteins such as PI3K and

Abbreviations: IGF, insulin-like growth factor; IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3-kinase; USP, ubiquitin-specific protease; SILAC, stable isotope labeling using amino acids in cell culture; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

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Grb2, and the protein complex formation triggers the activation of the downstream PI3K-Akt pathway and MAPK pathway, leading to the induction of IGF action [6]. We have shown that IRSs form high-molecular-mass complexes (IRSomes) [7], and other post-translational modifications and protein interactions of IRSs modulate IRS-mediated IGF/insulin signaling [8,9].

Ubiquitination plays important roles in the regulation of IGF/insulin signaling [10]. Several ubiquitin ligases ubiquitinate IRSs and induce their proteasomal degradation thereby attenuating IGF/insulin signaling, which results in muscle atrophy as well as insulin resistance [11–15]. Recently, we have reported that ubiquitin ligase Nedd4 associates with IRS-2 and induces mono-ubiquitination of IRS-2, which promotes IRS-2 recruitment to the plasma membrane, and enhances IRS-2 tyrosine phosphorylation by IGF-I receptor [16]. Our subsequent study suggested that this novel mechanism is utilized for thyroid stimulating hormone (TSH)-dependent enhancement of IRS-2 tyrosine phosphorylation and potentiation of IGF-I mitogenic action in thyrocytes, and for the acceleration of zebrafish embryonic growth [16]. It was also indicated that, in some types of cancer cells, excess activation of this mechanism by Nedd4 overexpression contributes to abnormal cell proliferation [16].

In general, protein ubiquitination levels are determined by the balance of ubiquitin conjugation reaction by ubiquitin ligases and the removal reaction by deubiquitination enzymes. Although we had reported that ubiquitin-specific protease 7 (USP7) deubiquitinates IRSs and prevents their proteasomal degradation [17], it remains unclear which deubiquitination enzyme(s) counteract IRS-2 ubiquitination by Nedd4. In this study, we identified USP15 as a novel deubiquitination enzyme for IRS-2, and found that USP15 antagonizes the effect of Nedd4 on IRS-2, thereby attenuating IGF-I signaling.

2. Material and methods

2.1. Antibodies, siRNAs and other materials

An anti-IRS-2 antibody raised in house [7] was used for immunoprecipitation, and anti-IRS-2 antibody (#sc-8299, Santa Cruz, CA, USA) was used for immunoblotting. Other antibodies used were as follows: anti-FLAG antibody (clone M2), and anti-FLAG M2 antibody-conjugated agarose beads, anti-phosphotyrosine antibody (clone PY20) from Sigma-Aldrich (St Louis, MO, USA); anti-myc antibody (clone 9E10), anti-ubiquitin antibody (clone P4D1), anti-IGF-IR antibody (#sc-713), anti-Akt antibody (#sc-8312) from Santa Cruz biotechnology; anti-USP15 antibody (#A300-923A) from Bethyl Laboratories (Montgomery, TX, USA); anti-Nedd4 antibody (#07-049) from Millipore (Billerica, MA, USA); anti-HSP70 antibody (#EP1007Y) from Abcam (Cambridge, UK); anti-phospho-Akt (threonine 308) antibody (#2965), anti-phospho-Akt (serine 473) antibody (#4060), anti-Erk1/2 antibody (#9102), anti-phospho-Erk1/2 antibody (#9101) from Cell Signaling (Danvers, MA, USA). siRNAs against human USP15 were designed as follows: #1, 5′-GAUGAUACCAGGCAUAUAAdTdT-3′; #2, 5′- GUCCCCAGGUGCAUCCAAUdTdT-3′. These siRNAs and a universal negative control RNA were synthesized by Nippon EGT (Toyama, Japan). siUSP15#1 was used in Figs. 2b and 4a, and siUSP15#2 was used in Fig. 4a and b. Recombinant human IGF-I was donated by Dr. Toshiaki Ohkuma (Fujisawa Pharmaceutical Co., Osaka, current Astellas Pharma Inc., Tokyo, Japan).

2.2. Plasmid construction

Human USP15 cDNA was amplified by PCR from Huh7 hepatocyte cDNA library. Site-directed mutagenesis was carried out by standard PCR-based method. USP15 cDNA was subcloned into pmyc-CMV5 (gift from Dr. Jun Nakae, Keio University, Tokyo, Japan) for N-terminal myc-tagged protein expression. The construction methods for other expression plasmids were previously described [16].

2.3. Cell culture and transfection

HEK293 cells, HEK293T cells and prostate cancer PC-3 cells [gifts from Dr. Akio Matsubara (Hiroshima University)] were maintained in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, Biomol, Nuaille, France) and antibiotics. HEK293 cells were transfected with plasmids and/or siRNA using Lipofectamine 2000 (Life technologies, Gaithersburg, MD, USA). HEK293T cells were transfected using polyethylenimine (Linear, Molecular Weight 25,000) (Polysciences, Warrington, PA, USA) [16], or using X-tremeGENE HP (Roche, Basel, Switzerland) in SILAC experiments. PC-3 cells were transfected with siRNA using Lipofectamine RNAiMAX (Life technologies). One day (HEK293 cells and HEK293T cells) or two days (PC-3 cells) after transfection, cells were serum-starved for 18 h unless otherwise specified. Cells were then stimulated with or without IGF-I, followed by indicated biochemical experiments.

2.4. SILAC and quantitative MS analysis of IRS-2-associated proteins

For SILAC (Stable Isotope Labeling using Amino Acids in Cell Culture), “light” medium was prepared using DMEM without L-Arginine and L-Lysine (Wako, Osaka, Japan), L-Arginine-$^{12}C_6$ (final conc., 100 mg/l), L-Lysine-$^{12}C_6$ (final con., 100 mg/l), and dialyzed FBS (Thermo Fisher Scientific, Rockford, IL, USA, final conc., 10%). Preparation of “heavy” medium was same except that L-Lysine-$^{13}C_6$ (Wako) was used. HEK293T cells were cultured in the “light” or “heavy” medium for 1 week, and then transfected with plasmids encoding FLAG-IRS-2-Ub or FLAG-IRS-2-Ub^{I44A}. Cells were serum-starved for 18 h, and then lysed with lysis buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM NaF, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 500 μM Na_3VO_4, 10 mg/ml *p*-nitrophenyl phosphate, protease inhibitor cocktail (Sigma-Aldrich, #P8340)] supplemented with 2 mM N-ethylmaleimide. Lysates were subjected to immunoprecipitation with anti-FLAG antibody-conjugated beads. 3xFLAG peptides (Sigma-Aldrich) were used for the competitive elution. FLAG-IRS-2-Ub immunoprecipitates and FLAG-IRS-2-Ub^{I44A} immunoprecipitates were mixed together, and subjected to acetone precipitation. The precipitates were diluted with 100 μl of trypsin solution [10 ng/μl Trypsin Gold Mass Spectrometry Grade (Promega, Madison, WI, USA), 50 mM ammonium bicarbonate, 0.01% RapiGest SF (Waters, Milford, MA, USA)], and incubated at 37 °C for 12 h. The sample was desalted using GL-Tip SDB and GL-Tip GC (GL Sciences, Tokyo, Japan), and then subjected to nano-liquid chromatography column (EASY-nLC 1000, Thermo Fisher Scientific) coupled to Q Exactive mass spectrometer (Thermo Fisher Scientific). The LC-MS/MS operation procedure was described elsewhere [18]. Data was analyzed by Proteome Discoverer software (Thermo Fisher Scientific).

2.5. Immunoprecipitation and immunoblotting

Cells were lysed with lysis buffer described above. Immunoprecipitation and immunoblotting were performed as described previously [16]. Densitometric analysis was carried out using ImageJ 1.43u program (National Institutes of Health, Bethesda, MD, USA).