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Over-accumulation of nuclear IGF-1 receptor in tumor cells requires elevated expression of the receptor and the SUMO-conjugating enzyme Ubc9

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ABSTRACT

The insulin-like growth factor 1 receptor (IGF-1R) plays crucial roles in tumor cell growth and is overexpressed in many cancers. IGF-1R's trans-membrane kinase signaling pathways have been well characterized. Very recently, we showed that SUMOylation mediates nuclear translocation of the IGF-1R, and that nuclear IGF-1R (nIGF-1R) binds to enhancer regions and activates transcription. We identified three lysine residues in the β-subunit of the receptor and that mutation of these blocks nuclear translocation and gene activation. Furthermore, accumulation of nIGF-1R was proven strongly dependent on the specific SUMO-conjugating enzyme Ubc9. Here we show that nIGF-1R originates *solely* from the cell membrane and that phosphorylation of the core tyrosine residues of the receptor kinase is crucial for nuclear accumulation. We also compared the levels of nIGF-1R, measured as nuclear/membrane ratios, in tumor and normal cells. We found that the breast cancer cell line MCF-7 has 13-fold higher amounts of nIGF-1R than breast epithelial cells (IME) which showed only a small amount of nIGF-1R. In comparison, the total expression of IGF-1R was only 3.7- higher in MCF-7. Comparison of several other tumor and normal cell lines showed similar tumor cell over-accumulation of nIGF-1R, exceeding the total receptor expression substantially. Ectopic overexpression (>10-fold) of the receptor increased nIGF-1R in IME cells but not to that high level as in wild type MCF-7. The levels of Ubc9 were higher in all tumor cell lines, compared to the normal cells, and this probably contributes to over-accumulation of nIGF-1R. Over-accumulation of nIGF-1R may contribute to deregulated gene expression and therewith play a pathophysiological role in cancer cells.

1. Introduction

The insulin-like growth factor 1 receptor (IGF-1R) is a receptor tyrosine kinase (RTK) that exerts pleiotropic effects after extracellular stimulation with IGF-1or IGF-2. Ligand binding triggers auto-phosphorylation of tyrosine residues, which subsequently activates a series of intracellular downstream signaling cascades like the phosphatidyl inositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. IGF-1R promotes several functions as cell proliferation, anti-apoptosis, angiogenesis, differentiation, and development [1–5]. In particular, IGF-1R has proven important in tumor cell growth and in tumor transformation, and IGF-1R is commonly overexpressed or highly activated in cancer cells and tissues [6,7].

The mechanisms underlying the fundamental roles of IGF-1R in cancer biology are, however, still not fully understood. Actually, IGF-1R shares the known major signaling pathways with other RTKs, like the insulin receptor (IR). Probably, IGF-1R promotes alternative signaling and actions.

Recently, we reported that IGF-1R undergoes SUMOylation and that this modification induces nuclear accumulation of the receptor [8]. Furthermore, nuclear IGF-1R (nIGF-1R) was demonstrated to bind to putative enhancer sites in the genomic DNA and to increase transcription [8]. SUMO1 modification occurred at three evolutionarily conservative lysine residues (K1025, K1100, and K1120) of the β-subunit. When these lysines were mutated the IGF-1R failed to get nuclear and to activate transcription [8]. Also, modification of the levels of Ubc9, the specific SUMO-conjugating enzyme [9], drastically changed the nuclear content of IGF-1R [8]. Our study implicated an alternative signaling of the IGF-1R with potential roles in cell regulation, and it also raised the question for functional roles in cancer cells. Nuclear translocation of IGF-1R in cancer cells was very recently highlighted [10].

In this study we compared nIGF-1R accumulation in neoplastic and normal cells in relation to expression of IGF-1R and Ubc9. Furthermore, we sought to clarify the principle shuttling pathway(s) of nIGF-1R.

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2. Materials and methods

2.1. Reagents

Figitumumab (CP-751,871) was a gift from Pfizer (Pfizer Research Labs, La Jolla, CA). Polyclonal IGF-1Rβ antibody, Na, K-ATPase antibody, EEA1 antibody, Calnexin antibody, and polyclonal SUMO-1 antibody were purchased from Cell Signaling Technology. Mouse monoclonal antibodies against Ubc9 were purchased from BD Biosciences. Anti-hemagglutinin (anti-HA) from Roche and monoclonal EGFP-antibody from Clontech were used. Polyclonal IGF-1Rα (N-20) antibody, Polyclonal Lamin A (H-102) antibody, and Polyclonal GAPDH antibody were purchased from Santa Cruz Biotechnology Inc. Magnetic Protein G coupled dynabeads, NuPAGE® Bis-Tris gels for western blot were purchased from Invitrogen. All other reagents unless stated otherwise were from Sigma.

2.2. Cell cultures

Human breast cancer cells MCF-7 and telomerase reverse transcriptase-immortalized human mammary epithelial (IME) cells were cultured in standard media as described elsewhere [11].

Three mouse embryonic fibroblast (MEFs) derivatives P6 (overexpressing human normal IGF-1R), TM (*IGF1R*$^{-/-}$ cells transfected with human IGF1R with Y1131F, Y1135F, and Y1136F mutations), and R-56 (stably transfected with IGF-1R with a truncated C-terminal domain) were cultured in DMEM (4.5 g/l glucose with glutamine) supplemented with 5% FBS and 200 μg/mL G418, 10% FBS and 250 μg/mL G418, 10% FBS and 2.5 μg/mL Puromycin, respectively. The P6, TM, and R-56 cells were kind gifts from Dr. Renato Baserga (Thomas Jefferson University, Philadelphia, PA). The mutations of *IGF1R* in TM and R-56 cells were confirmed by sequence analysis before the study.

LP1 (human multiple myeloma cells) [12], RPMI-8226 (human multiple myeloma cells) [13], DFB (human melanoma cells) [8], ES1 (human Ewing's sarcoma cells) [14], BJ (hTERT) (human immortalized fibroblasts) [15], rSMC (rat smooth muscle cells) [16], and early passages of human diploid fibroblasts (HDF) were cultured under standard conditions as described elsewhere.

2.3. Plasmids and transient transfections

The human wild type (WT) IGF-1R cDNA plasmid was a kind gift from Dr. Renato Baserga (Thomas Jefferson University, Philadelphia, PA). The triple SUMO mutant (TSM) IGF-1R was constructed using QuickChange site-directed mutagenesis kit (Stratagene, San Diego, CA, USA) to introduce three point mutations at K1025R, K1100R, and K1200R into the WT *IGF1R* plasmid causing inhibition of SUMOylation of the receptor [8]. All of these mutations were confirmed by DNA sequencing.

One day before transfection, we plated cells in growth medium without antibiotics so that cells will be 90–95% confluent at the time of transfection. IME cells were transfected with plasmids DNA (WT/IGF-1R, TSM, and Mock) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Medium may be changed after 5–6 h and cells are harvested at 48 h for blotting analyses. Also, IME cells were transfected with *HA-UBC9* plasmid.

2.4. Subcellular fractionation

Cytoplasmic, membranous, and nuclear proteins were sequentially isolated using the Qproteome Cell Compartment Kit according to the manufacturer's protocol (Qiagen, Chatsworth, CA). Firstly, Extraction Buffer CE1 was added to cells and selectively disrupts the plasma membrane without solubilizing it, resulting in the isolation of cytosolic proteins. Plasma membrane and compartmentalized organelles including endoplasmic reticulum (ER) remain intact. Then, Extraction Buffer CE2 was used to solubilize the plasma membrane as well as organelle membranes except the nuclear membrane. After centrifugation, the supernatant contains membrane proteins and proteins from ER and mitochondria. In the final step, all soluble and membrane-bound nuclear proteins were extracted using Extraction Buffer CE3. The efficiency of the cell fractionation was controlled using marker proteins for cell nuclei (Lamin), plasma membranes (Na, K-ATPase), early endosomes (EEA1), and ER (Calnexin).

2.5. SDS–PAGE and Western blotting

In order to prepare whole cell lysate, cells were incubated with pre-cold PBS-TDS lysis buffer (1× PBS, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 8.0) on ice for 30 min, and then were centrifuged for 15 min at 12,000 rpm 4 °C. The protein concentration was determined by the Bradford method. Equal amounts of protein were solubilized in sample buffer and electrophoresed on denaturing SDS–polyacrylamide gel. The proteins were then transferred to nitrocellulose membranes (Hybond™-C-Super, Amersham, UK) and blotted with the indicated antibodies. This was followed by washes and incubation with an HRP conjugated secondary antibody (ImmunoPure antibody, Pierce) and detection with Hyperfilm-ECL, Amersham, UK.

2.6. Immunoprecipitation

The whole cell lysate or fractionated cell lysate were prepared (see Section 2, Western blotting and subcellular fractionation). The samples were pre-cleared 1 h with Dynabeads (Invitrogen) without antibody. The protein concentration of the resulting supernatant was determined by the bicinchoninic acid assay (BCA protein assay kit, Pierce) and the same amount of protein were incubated with Dynabeads protein G previously attached to IGF-1Rα antibody (Calbiochem) over night at 4 °C. Fractions prepared with Cell Compartment Kit (Qiagene) were diluted 1:2 with Immunoprecipitation (IP) dilution buffer (0.5% Triton-x-100, 0.5% Doc Deoxycholic acid, 150 mM NaCl, 20 mM Tris pH 7.5, 30 mM NaPyroPhosphate, 20 mM NEM) and the IP was carried out as explained above. The magnetically separated complex was washed three times with whole cell lysis buffer, and were boiled in SDS sample loading buffer for 10 min, separated by SDS–PAGE and further was analyzed by Western blotting.

3. Results

3.1. Nuclear IGF-1R originates from cell surface and is dependent on kinase activity

The breast cancer cell line MCF-7 is widely used in IGF-1 research. First, we investigated the effects of figitumumab (CP-751,871), an IGF-1R specific humanized monoclonal antibody [17], on IGF-1R expression in MCF-7 cells. As shown in Fig. 1A, the antibody caused a time-dependent (0–24 h) downregulation of IGF-1R, as assessed by Western blotting. To determine the effects on a subcellular level, cells were fractionated in a parallel experiment. The major part of the receptors is localized in the membranes, but a substantial portion of it is nuclear (Fig. 1B). Treatment with CP-751,871, which is unable to penetrate the cell membrane, caused a time-dependent and equal decrease of membrane and nuclear receptors. In contrast, the levels of the pro-receptors remained unaffected (Fig. 1B). Lamin, used as a nuclear marker, was detectable only in the nuclear fractions. As shown in