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C-Src-mediated phosphorylation of δ-catenin increases its protein stability and the ability of inducing nuclear distribution of β-catenin

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ABSTRACT

Although δ-catenin was first considered as a brain specific protein, strong evidence of δ-catenin overexpression in various cancers, including prostate cancer, has been accumulated. Phosphorylation of δ-catenin by Akt and GSK3β has been studied in various cell lines. However, tyrosine phosphorylation of δ-catenin in prostate cancer cells remains unknown. In the current study, we demonstrated that Src kinase itself phosphorylates δ-catenin on its tyrosine residues in prostate cancer cells and further illustrated that Y1073, Y1112 and Y1176 of δ-catenin are predominant sites responsible for tyrosine phosphorylation mediated by c-Src. Apart from c-Src, other Src family kinases, including Fgr, Fyn and Lyn, can also phosphorylate δ-catenin. We also found that c-Src-mediated Tyr-phosphorylation of δ-catenin increases its stability via decreasing its affinity to GSK3β and enhances its ability of inducing nuclear distribution of β-catenin through interrupting the integrity of the E-cadherin. Taken together, these results indicate that c-Src can enhance the oncogenic function of δ-catenin in prostate cancer cells.

1. Introduction

Src family kinases (SFKs) are the largest family of nonreceptor protein tyrosine kinases and responsible for signal transduction during many cellular activities [1]. All SFK members share similar structures. Each kinase composes of four Src homology (SH) domains and a unique amino-terminal domain. On the basis of overall homology, SFKs can be classified into two subfamilies. One is the Src subfamily including Src, Yes, Fyn and Fgr. The other is the Lyn subfamily containing Lyn, Hck, Lck and Blk. In addition, there are three SFK related kinases, Brk, Frk, and Srm [2]. Among these kinases, Src, Fyn and Yes are ubiquitously expressed in all cells while other members are tissue specific. As the most wildly studied kinase among SFKs, Src has been implicated in a variety of malignancies [3], including prostate cancer [4]. Other than Src, Fgr [5] and Lyn [6] have also been found to be involved in prostate cancer.

Src has been proven to provide oncogenic signals for cell survival, mitogenesis, epithelial-mesenchymal transition (EMT), invasion, angiogenesis, and metastasis [7]. It has also been known to interact with p120catenin (p120ctn) which was originally identified as a major substrate of oncogene v-Src and as an important regulator of cadherin–catenin complex mediating cell–cell adhesion [8]. So far, 8 tyrosine sites on p120ctn have been identified to be phosphorylated by Src [9]. However, the functional consequences of each specific modification are not yet clear. Although it has been found that the differential phosphorylation of tyrosine 112, 217 and 228 by Fyn and Src kinases modulates the ability of p120ctn to bind and inhibit Rho A [10], it is not fully known how Src-induced p120ctn-phosphoylation modifies cadherin functions.

δ-catenin is a member of the p120ctn subfamily of armadillo proteins and was first identified through its interaction with presenilin-1 [11]. Although δ-catenin was first considered as a brain specific protein, lately, its overexpression has been discovered in a variety of cancer tissues, including prostate [12–14], lung [15–18], ovarian [19], brain [20] and colorectal tumors [21]. Furthermore, strong evidence of δ-catenin overexpression has been accumulated in prostate cancer. It has been reported that δ-catenin is overexpressed at both mRNA and protein levels in prostate cancer [12,13]. It has also been demonstrated that δ-catenin is accumulated in the urine of prostate cancer patients [14]. The function of overexpressed δ-catenin in prostate cancer has been extensively studied. It has been reported that δ-catenin promotes prostate cancer cell growth and progression by altering the cell cycle and profiles of survival genes [22]. In our previous study, we found that δ-catenin induces E-cadherin processing and activates the β-catenin signaling pathway in prostate cancer [23]. We also demonstrated that δ-catenin promotes angiogenesis through stabilizing HIF-1α to activate VEGF in the CWR22Rv-1 prostate cancer cell line [24]. These studies suggest the important role of δ-catenin in prostate cancer. However, δ-catenin has

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been identified as a potential suppressor of anchorage-independent growth through siRNA-based genetic screening of human mammary epithelial cells [25]. These contrary observations led us to question if modification of δ-catenin, especially tyrosine phosphorylation, can change its biological functions.

Unlike p120ctn, there have been only few studies about tyrosine phosphorylation of δ-catenin since it is a relatively new member in the p120ctn subfamily of armadillo proteins. Lu et al. found that δ-catenin can be phosphorylated on Y289 and Y429 by Abl kinase [26]. Maria CM et al. discovered that δ-catenin can interact with the SH3 domain of both Fyn and Lck and be phosphorylated by Fyn at its C-terminus [27]. They also reported that tyrosine phosphorylation of δ-catenin can mediate switching of its role between inducing neurite elongation and inducing neurite branching. Although the overexpression of δ-catenin in prostate cancer has been proven, whether tyrosine phosphorylation of δ-catenin occurs in prostate cancer cells remains unknown.

Therefore, in this study, we demonstrated that Src kinase itself phosphorylates δ-catenin on its tyrosine residues in prostate cancer cells and further proved that Y1073, Y1112 and Y1176 of δ-catenin are predominant sites responsible for tyrosine phosphorylation mediated by c-Src. In addition to c-Src, Fgr, Fyn and Lyn were also found to phosphorylate δ-catenin. Furthermore, c-Src-mediated Tyr-phosphorylation of δ-catenin was found to increase its stability via decreasing its affinity to GSK3β and enhance its ability of inducing nuclear distribution of β-catenin through interrupting the integrity of the E-cadherin complex.

2. Materials and methods

2.1. Plasmids

The constructs of δ-catenin wild type (WT), ΔN85-325, 1-1040 in pEGFP-C1 have been previously described [43]. The deletion constructs of 1-690, 690-1040, 1-1070, 1-1140, 1-1170 and 1-1120 were generated by PCR amplification and cloned into pEGFP-C1 vector. The construction of mutants Y1176F, Y1179F, Y1189F, Y1176/1179/1189F, Y1073(1-1140), Y1112F(1-1140) and Y1121F(1-1140) was generated by site-directed mutagenesis. The constructs of pCMV5 RF-Src [dominant-negative (K295R, Y527F)] and Src were kindly provided by Joan Brugge (Harvard Medical School). The constructs of HM-Fyn, HM-Fgr and MH-Lyn were generously gifted by Nacksung Kim (Chonnam National University).

2.2. Antibodies

Antibodies were purchased from commercial companies as follows: anti-δ-catenin (#611537), anti-E-cadherin (#610182, BD Bioscience); anti-GFP (#G1544), anti-β-actin (#A5441), anti-β-catenin (#C2206), and anti-α-tubulin (#T9026, Sigma), anti-lamin B (SC-6216), anti-py20 (SC-508) and anti-E-cadherin (SC-7080, Santa Cruz); anti-Src (#05-184), anti-Fyn (#4023), anti-Fgr (#2755), anti-Lyn (#2732), anti-myc (#2276) and anti-GSK3α/β(#5676, Cell Signaling). HA epitope was detected using media from 12CA5 hybridoma.

2.3. Cell culture and transfection

MEF, NIH/3T3 and Bosc23 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO_2. CWR22Rv-1 (Human prostate cancer cell line) cells were grown in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO_2. Rv/δ and Rv/C cells derived from CWR22Rv-1, overexpressing δ-catenin and GFP respectively, were maintained in RPMI supplemented with 10% FBS, 1% penicillin/streptomycin and G418 (Sigma, St Louis, MO) 125 μg/ml at 37 °C with 5% CO_2.

Bosc23 cells were transfected using calcium phosphate while other cells were transfected using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

2.4. Sodium orthovanadate treatment

Sodium orthovanadate obtained from Sigma was dissolved in distilled water to make a stock concentration of 20 mM. The stock solution of sodium orthovanadate was added into warm media to make a final concentration of 40 μM followed by gentle mixing. Following this, the media in the culture dishes were replaced with the media containing sodium orthovanadate and cells were kept in this media for 24 h.

2.5. Immunoblotting and immunoprecipitation

Immunoblotting and immunoprecipitation were performed as previously described [28]. Lysates were incubated with primary antibodies for 16 h at 4 °C and pulled out with protein G sepharose (GE healthcare, Uppsala, Sweden) for 3 h. The immunoprecipitated proteins were eluted at 95 °C for 2 min with 15 μl of 2× sample buffer (0.1 M Tris–HCl, pH6.8, 0.2 M DTT, 4% SDS, 20% glycerol, 0.2% bromophenol blue, 1.43 M β-mercaptoethanol) and analyzed by immunoblotting.

2.6. Purification of cell surface proteins by biotinylation

Biotinylation was performed as previously described [23]. Briefly, transfected Rv/δ and Rv/C cells were cultured in 100 mm dishes and were collected to perform further experiment when cells' density was near confluence. Specifically, cells were gently washed and collected in 1.5 microcentrifuge tubes using ice-cold reaction buffer (0.1 M phosphate, 0.15 M NaCl, pH 8.0). Cell surface proteins were labeled with Biotin by incubating the tubes with 0.5 mM Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL) at room temperature for 30 min. Next, the cells were lysed in MLB lysis buffer followed by centrifugation at 13,200 rpm for 15 min at 4 °C. After measuring the protein concentration of each sample, equal amounts of protein lysates were explored to pull-down with streptavidin-agarose beads (Pierce, Rockford, IL). This process resulted in purification of the membrane proteins. Next, the purified proteins were subjected to immunoblotting. The biotinylated plasma membrane localized E-cadherin was then detected by immunoblotting with an E-cadherin antibody.

2.7. Cell fractionation

The Subcellular Protein Fractionation Kit for Cultured Cells (78840, Thermo Scientific) was explored to separate cytoplasmic, membrane and nuclear protein extracts. Briefly, cells were harvested with trypsin-EDTA and centrifuged at 500 ×*g* for 5 min. The supernatant was then removed carefully, and the cell pellets were washed with PBS followed by centrifugation at 500 ×*g* for 2–3 min. The supernatant was discarded carefully again to leave cell pellets as dry as possible. Next, specific buffers in the Kit were added into the tubes containing the cell pellets in certain order according to the protocol.

Protein extracts from different compartments of cells were subjected to immunoblotting. Lamin B antibody was used as a nuclear protein marker while α-tubulin antibody and E-cadherin antibody were used as cytoplasmic and membrane protein markers, respectively.

2.8. Primary cortical neurons culture and siRNA transfection

Cortical neurons were prepared from E14 C57BL/6 embryos (Daehan Biolink, Daejeon, Korea). Cerebral cortices were removed from the embryos, partially dissociated in PBS and incubated with 0.25% trypsin in HBSS for 20 min at 37 °C. The trypsin was inactivated by adding FBS (Gibco, Gaithersburg, MD) and the tissue was triturated with a sterile constricted Pasteur pipette. Cortical neurons were seeded at 1×10^6 cells/well in 24-well plates pre-coated with 100 μg/ml poly-D-lysine (Sigma, St. Louis, MO). Cortical neurons were maintained in Neurobasal Media (Invitrogen, Carlsbad, CA) supplemented with B27