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Paxillin, a novel controller in the signaling of estrogen to FAK/N-WASP/Arp2/3 complex in breast cancer cells

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ABSTRACT

Breast cancer is the major cause of cancer-related death in women. Its treatment is particularly difficult when metastasis occurs. The ability of cancer cells to move and invade the surrounding environment is the basis of local and distant metastasis. Cancer cells are able to remodel the actin cytoskeleton, which requires the recruitment of numerous structural and regulatory proteins that modulate actin filaments dynamics, including Paxillin or the Neural Wiskott-Aldrich Syndrome Protein (N-WASP). We show that 17-β estradiol (E2) induces phosphorylation of Paxillin and its translocation toward membrane sites where focal adhesion complexes are assembled. This cascade is triggered by a Gαi1/Gβ protein-dependent signaling of estrogen receptor α (ERα) to c-Src, focal adhesion kinase (FAK) and Paxillin. Within this complex, activated Paxillin recruits the small GTPase Cdc42, which triggers N-WASP phosphorylation. This results in the redistribution of Arp2/3 complexes at sites where membrane structures related to cell movement are formed. Recruitment of Paxillin, Cdc42 and N-WASP is necessary for cell adhesion, migration and invasion induced by E2 in breast cancer cells. In parallel, we investigated whether Raloxifene (RAL), a selective estrogen receptor modulator (SERMs), could inhibit or revert the effects of E2 in breast cancer cell movement. We found that, in the presence of E2, RAL acts as an ER antagonist and displays an inhibitory effect on estrogen-promoted cell adhesion and migration via FAK/Paxillin/N-WASP. Our findings identify an original mechanism through which estrogen regulates breast cancer cell motility and invasion via Paxillin. These results may have clinical relevance for the development of new therapeutic strategies for cancer treatment.

1. Introduction

Breast cancer is the primary cause of cancer-related death in women. About one out of eight women develops breast cancer at some stage of her life (Torre et al., 2015). Metastasis is a complex process characterized by detachment of cells from the primary tumor followed by their dissemination through the blood vessels to distant organs, extravasation into the parenchyma of tissues and formation of secondary tumors at distant sites. The latter is the principal cause of death in cancer patients, which highlights the need for new therapeutic strategies (Liang and Shang, 2013; Mezi et al., 2012).

Sex steroid hormones play a key role in breast cancer development, progression and metastasis (Liang and Shang, 2013; Folkerd and Dowsett, 2010). Prolonged treatment with estrogen, such as late menopausal or postmenopausal hormone replacement therapy, is associated with a greater risk of developing breast cancer (Horwitz, 2008; Ma et al., 2006; Verkooijen et al., 2009). However, the effects of estradiol on breast tumor cell motility or invasion are poorly understood.

Cell migration is required for cancer spread, invasion and metastasis. It is a complex multistep process that involves protrusion of the leading edge of the cell, formation of adhesion complexes, myosin/actin-mediated cell contraction, and the release of adhesions at the cell rear (Deakin and Turner, 2008; Vinzenz et al., 2012). We have recently shown that 17β-estradiol (E2) promotes the depolymerization of actin fibers and the reassembly toward the cell membrane edge, leading to the formation of cortical actin complexes implicated in the generation of the breast cancer cell

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locomotive force (Flamini et al., 2009; Sanchez et al., 2010; Sanchez et al., 2009; Simoncini et al., 2006). Dynamic adhesion involves the coordinated action of different proteins, including the proteins kinase c-Src, and focal adhesion kinase (FAK). The Src/FAK complex interacts with Paxillin and activates it by phosphorylation on Tyr^{31} and Tyr^{118} (Zaidel-Bar et al., 2007). Paxillin is a scaffolding protein that serves as a platform for the recruitment of several regulatory and structural proteins that modulate the dynamic changes in cell adhesion, mediate the control in the assembly and disassembly of the focal adhesion complexes, and as well as cytoskeletal reorganization promoting the cell migration and invasion process (Deramaudt et al., 2014).

The Neural Wiskott-Aldrich syndrome protein (N-WASP) is one of the many controllers of the actin cytoskeleton. N-WASP acts as a scaffolding complex, relaying signals from small GTPases such as Cdc42 to the Arp2/3 complex (Rohatgi et al., 1999). Actin nucleation by the Arp2/3-complex appears to be fundamental for the rapid formation of an actin network at the leading edge of the cell (Takenawa and Suetsugu, 2007). Recent studies have demonstrated that Paxillin may allow the recruitment of N-WASP and modulate the activation of the Arp2/3 complex (Lettau et al., 2010; Oda and Eto, 2013; Dummler et al., 2009; Jessick et al., 2013). This activation leads to branching and actin polymerization, allowing the formation of specialized membrane structures that promote cell movement (Padrick and Rosen, 2010; Frugtniet et al., 2015).

The aim of the present study was to identify the molecular basis of the actions of estrogen on breast cancer cell morphology. In particular, we wanted to identify whether these actions may require the regulation of the actin cytoskeleton via Paxillin to the N-WASP-Arp2/3 complex, and to characterize the intracellular cascades that may be recruited during this signaling.

2. Materials and methods

2.1. Cell cultures and treatments

MCF-7, T-47D and MDA-MB-231 Human breast cancer cells were obtained from the American Type Culture Collection. MCF-7 cells were cultured in minimal essential medium (MEM) with L-glutamine (2 mM) and 10% fetal bovine serum (FBS). T-47D cells were grown in RPMI 1640 supplemented with L-glutamine (2 mM), 10% fetal bovine serum. MDA-231 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 10% penicillin/streptomycin. Before treatments, breast cancer cells were kept for 24 h in medium containing steroid-deprived FBS. To investigate non-genomic effects, cancer cells remained in medium without FBS for 8 h. 17β-estradiol (E_2) and pertussis toxin (PTX) were purchased from Sigma-Aldrich (Saint-Louis, MO, USA); ICI 182,780 (ICI) was from Tocris Cookson (Avonmouth, UK); 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo(3,4-d)pyrimidine (PP2) was obtained from Calbiochem (EMD Biosciences, Germany); FAK inhibitor 14 (FAKi, sc-203950A) and Arp2/3 inhibitor (CK666, sc-361151) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Whenever an inhibitor was used, the compound was added 45 min before starting the active treatments.

2.2. Immunoblottings

Cell lysates were separated by SDS-PAGE. Antibodies used were: Paxillin (sc-31010), p-Paxillin (Tyr^{118}), p-FAK (Tyr^{397}), ERα (H-184), ERβ (N-19), Gαi1 (R4), Gβ1 (C-16), Cdc42 (B-8), actin (Santa Cruz Biotechnology, Santa Cruz, CA); Phospho-Rac1/Cdc42 (Ser^{71}) (Cell Signaling Technology); FAK, p-FAK (Y^{397}) (Transduction Laboratories, Lexington, KY, USA); N-WASP (ab32707), p-N-WASP ($Ser^{484/485}$) (Chemicon International, Millipore). Primary and secondary antibodies were incubated with the membranes using standard techniques. Immunodetection was carried out using enhanced chemiluminescence and was recorded with a quantitative digital imaging system (Chemidoc XRS with Image Lab, Bio-Rad, Hercules, CA, USA).

2.3. Cell inmunofluorescence

Human breast cancer cells were grown on coverslips and exposed to different treatments. Cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton for 5 min. Blocking was performed with 3% bovine serum albumin for 30 min. Cells were incubated with antibodies against p-Paxillin (Tyr^{118}, A-5); N-WASP (ab32707) (Chemicon International, Millipore); Arp3 (Transduction Laboratories, Lexington, KY, USA) overnight at 4 °C. Phospho-Paxillin and Arp3 were incubated with a $fluorescein^{488}$ (FITC)-conjugated goat anti-rabbit/mouse secondary antibody (1:200; Vector Laboratories). N-WASP was incubated with anti-mouse-$Dylight^{594}$ (1/125) secondary antibody. Texas Red-phalloidin (Sigma-Aldrich) was added for 30 min. The nuclei were counterstained with 4-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Immunofluorescence was visualized using an Olympus BX41 microscope and recorded with a high-resolution DP70 Olympus digital camera.

For colocalization assays, MCF-7 cells were grown on coverslips and exposed to different treatments. Cells were incubated with pPaxillin with a $fluorescein^{488}$ (FITC)-conjugated goat anti-mouse secondary antibody (1:200; Vector Laboratories), N-WASP with a $DyLight^{594}$-conjugated goat anti-rabbit secondary antibody (1:200; Vector Laboratories) and FAK with a $DyLight^{594}$-conjugated goat anti-rabbit secondary antibody (1:200; Vector Laboratories). The nuclei were counterstained with 4-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). The immunostainings were examined with a confocal microscope (FV 1000; Olympus) with a Paplon 60 × lens.

2.4. Quantitative colocalization analysis

The colocalization analysis was made using the JACoP plugin for Image J software according to Bolte and Cordelieres (Bolte and Cordelieres, 2006). Z-Stack images of MCF-7 cells stained for both pPaxillin/FAK and pPaxillin/N-WASP were deconvolved. The channel-specific point spread functions were generated and the signal:noise ratio was adjusted until deconvolved images were free of pixel noise. The colocalization analysis was made using two correlation coefficients, Pearson (PCC) and Manders (MCC). For PCC calculation, the dependency of pixels in dual-channel images (green and red channels for FAK/N-WASP and pPaxillin, respectively) was measured by plotting the pixel gray values of two images against each other. These values were displayed in a pixel-distribution diagram (scatter plot), and a linear equation of the relation between the intensities of the two images was calculated by linear regression. A cross-correlation function (CCF) was obtained by plotting the corresponding PCC for each pixel shift (delta x) of the green image in the x direction relative to the red image. The real value of the PCC is estimated at delta $x = 0$. This value can range from 1 to −1, with 1 standing for complete positive correlation, −1 for negative correlation, and 0 for no correlation. The MCC is based on the PCC, with average intensity values being taken out of a mathematical expression (Manders et al., 1992). Two coefficients were obtained, MCC-M1 and MCC-M2, for the fraction of FAK and/or N-WASP overlapping with pPaxillin and for the