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ARF1 regulates adhesion of MDA-MB-231 invasive breast cancer cells through formation of focal adhesions

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ABSTRACT

Adhesion complex formation and disassembly is crucial for maintaining efficient cell movement. During migration, several proteins act in concert to promote remodeling of the actin cytoskeleton and we have previously shown that in highly invasive breast cancer cells, this process is highly regulated by small GTP-binding proteins of the ADP-ribosylation factor (ARF) family. These are overexpressed and highly activated in these cells. Here, we report that one mechanism by which ARF1 regulates migration is by controlling assembly of focal adhesions. In cells depleted of ARF1, paxillin is no longer colocalized with actin at focal adhesion sites. In addition, we demonstrate that this occurs through the ability of ARF1 to regulate the recruitment of key proteins such as paxillin, talin and FAK to ß1-integrin. Furthermore, we show that the interactions between paxillin and talin together and with FAK are significantly impaired in ARF1 knocked down cells. Our findings also indicate that ARF1 is essential for EGF-mediated phosphorylation of FAK and Src. Finally, we report that ARF1 can be found in complex with key focal adhesion proteins such as ß1-integrin, paxillin, talin and FAK. Together our findings uncover a new mechanism by which ARF1 regulates cell migration and provide this GTPase as a target for the development of new therapeutics in triple negative breast cancer.

1. Introduction

Progression of cancer from primary tumors to metastatic carcinomas requires that cancer cells detach from the initial tumor to invade their surrounding. Through the lymphatic or circulatory systems, cells can attain distant organs and tissues to initiate the formation of metastasis. The ability of a cell to migrate is therefore essential for that process. Cell migration requires the dynamic interaction between the cell and the extracellular matrix onto which it is attached and migrates. Adhesion is mediated by membrane-proximal protein complexes associated with integrins. These are transmembrane proteins that function to link the intracellular cytoskeletal components of the cells to the substratum on which cells are anchored. The intracellular domain of integrins acts as a scaffold for focal adhesion proteins, which formation and disassembly is controlled by more than 180 protein–protein interactions. These include cytoskeletal proteins such as paxillin and talin as well as enzymes such as FAK (Focal adhesion kinase) and small GTP-binding proteins [1,2]. Cell migration and invasiveness can be enhanced by numerous growth factors. For example, high expression of the epidermal growth factor receptor (EGFR) is a major cause of cancer development, progression, and is associated with a poor prognosis [3]. Basal or triple negative breast cancer cells, like MD Anderson-Mammary Breast cancer cell line-231 (MDA-MB-231), often express high EGFR levels, which is associated with a loss of sensitivity to hormonal therapies and/or a high probability of metastasis [4]. Increased activation of this receptor tyrosine kinase (RTK) has been correlated with, namely, enhanced survival, migration and invasiveness [5]. These three processes are mediated by intracellular signaling proteins of the Ras GTPase family [6]. Others and we have demonstrated that ADP-ribosylation factors (ARFs) are involved in the regulation of the biological effects induced by epidermal growth factor (EGF) [7–9]. Like all GTPases, ARFs are considered active when bound to GTP and inactive when bound to GDP. ARF1 and ARF6 are the best characterized from the six identified isoforms. ARF6 has been shown to localize to the plasma membrane, while ARF1 is classically associated with the Golgi and in some cell types can also be found associated with the plasma membrane [7,10,11]. These small GTP-binding proteins act as molecular switches to turn on signaling cascades associated with remodeling of the actin cytoskeleton, transformation of membrane lipids and vesicle formation [7,10,12,13].

Abbreviations: ARF, ADP-ribosylation factor; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; GAP, GTP activating protein; HCC70, Human Breast Cancer Cell Line 70; MDA-MB-231, MD Anderson-Mammary Breast cancer cell line-231; MDCK, Madin-Darby Canine Kidney; PBS, phosphate buffer saline; PI3K, phosphatidylinositol 3 kinase; PTK, protein-tyrosine kinase; RTK, receptor tyrosine kinase; SKBR3, Sloan Kettering human breast cancer cell line 3; siRNA, small interfering ribonucleic acids

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During migration, dynamic adhesion involves the coordinated action of various classes of molecules. The protein tyrosine kinase, focal adhesion kinase (FAK) is a non-receptor protein–tyrosine kinase (PTK), ubiquitously expressed in cytoplasm and involved in the formation of the adhesions complex [14]. FAK plays a role in the assembly and disassembly of the focal adhesion point [15]. It is involved in signal transduction and in the regulation of cell survival and motility triggered by the binding of integrins to the extracellular matrix [16,17]. Following the activation of EGFR and/or integrins, FAK is recruited to these receptors indirectly through C-terminal domain-mediated interactions with integrin-associated proteins such as paxillin and talin [18]. FAK activation leads to the autophosphorylation of Tyr397, which binds to the Src family kinases. In turn, Src phosphorylates FAK on Tyr 861 [19]. FAK then becomes the main anchoring point from which focal adhesions complex are formed [18]. Certain ARF GAPs, such as GIT and ASAP1 have been shown to interact with FAK and paxillin [20,21]. Futhermore, in Swiss 3T3 Fibroblasts, ARF1 has been reported to mediate the recruitment of paxillin to focal adhesion [22]. In addition, we showed that another ARF isoform, ARF6, is required for remodeling of the focal adhesion complex in endothelial cells [23]. Despite the studies conducted, the detailed mechanism through which ARF1 regulates adhesion in highly invasive breast cancer cells remains unknown. In this study, we report that ARF1 controls formation of focal adhesions complex. Using RNAi and a chemical inhibitor targeted to inhibit the expression or activation of this ARF isoform, we provide evidence that ARF1 acts to regulate key protein–protein interactions between ß1-integrin, paxillin, talin, and FAK. Furthermore, ARF1 expression and activation impacts the ability of FAK, as well as Src, to become phosphorylated. Finally, we show that ARF1 is an integral part of focal adhesion complex by demonstrating that this GTPase can also be found interacting with these key molecules.

By defining the key signaling proteins regulating adhesion, we will gain further insights into the mechanism by which tumor cells become motile and contribute to the progression and aggressiveness of breast cancer. The identification of proteins that orchestrate fundamental cellular responses such as migration will help us identify new molecular therapeutic targets for the treatment of highly invasive breast cancers for which therapeutical options remain limited.

2. Material and methods

2.1. Reagents and antibodies

Anti-paxillin, anti-FAK pY397 and anti-CD29 were purchased from BD Bioscience (Bedford, MA, USA). Anti-FAK pY861 polyclonal antibody, Lipofectamine 2000, Alexa Fluor 488-phalloidin and Rhodamine-phalloidin were purchased from Invitrogen (Burlington, ON, Canada). Anti-ARF1 was obtained from ProteinTech (Chicago, IL, USA). Anti-Pan-Actin, anti-EGFR, anti-Src pY416 and anti-Giantin were from Cell Signaling Technology (Danvers, MA, USA). Anti-FAK (A-17), anti-ARF6 (3A1) and anti-talin were purchased from Santa Cruz Biotech Inc. (Santa Cruz, CA, USA). All others products were from Sigma Aldrich Company (Oakville, ON, Canada). LM11 compound was synthesized by Dr Pierre Lavallée (Combinatorial Chemistry Laboratory, Université de Montréal, QC, Canada). GST-GGA3 was a gift from Dr. Jean-Luc Parent (Université de Sherbrooke, Sherbrooke, QC, Canada).

2.2. Cell culture and transfection

MDA-MB-231, Human Breast Cancer Cell Line 70 (HCC70) and Sloan Kettering human breast cancer cell line 3 (SKBR3) cells were obtained from Dr. Sylvie Mader (Université de Montréal, Montreal, QC, Canada). Cells were maintained at 37 °C, 5% CO_2, in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 10% penicillin/streptomycin. All cell culture reagents were purchased from Wisent Bioproducts (St-Bruno, QC, Canada). Transfection of small interfering ribonucleic acids (siRNAs), (72 h) was conducted using Lipofectamine 2000 from Invitrogen (Burlington, ON, Canada) according to the manufacturer's instructions. siRNA corresponding to human ARF1 (-GAACCAGAAGUGAACGCGA- was used in all experiments and -CGGCCGAGATCACAGACAAG- a second sequence, was used to confirm specificity of the effects of the siRNA), to a scrambled ARF1 siRNA sequence and human FAK (-GGACAUUAUUGGCCACUGU-) were synthesized by Thermo Science Dharmacon (Lafayette, CO, USA). ARF1-HA was a generous gift from Nicolas Vitale (Université de Strasbourg, Strasbourg, France). For rescue experiments, ARF1-HA was transfected 24 h after introduction of the ARF1 siRNA sequence #1.

2.3. Immunofluorescence

The cells were fixed with a phosphate buffer saline (PBS) solution containing 4% paraformaldehyde for 15 min at room temperature and then permeabilized with a solution of DMEM containing 0.05% saponin. The slides were incubated for 1 h with a primary antibody. After several washes, the plates were incubated for 1 h in the dark in the presence of an anti-mouse or anti-rabbit Alexa Fluor 488-antibody or Alexa-Fluor 568 (Invitrogen, Burlington, ON, Canada). They were then mounted onto slides using a solution of Aqua-mount (Fisher Scientific, Ottawa, ON, Canada) and observed using a confocal microscope (LSM510META, Carl Zeiss).

2.4. Migration assay

MDA-MB-231 cells were seeded onto collagen-coated (Sigma-Aldrich, Oakville, ON, Canada) Boyden Chambers (8 μm) (Corning, NY, USA) and treated with different doses of LM11 or vehicle. One hour later, cells were stimulated with 10 ng/ml EGF and incubated for 6 h. Cells were fixed using paraformaldehyde (4%) and stained with crystal violet (0.1% in 20% methanol, 16 h). Cells present in the upper chamber were removed with a cotton swab and cells that migrated were quantified in the lower chamber by counting.

2.5. Co-immunoprecipitation experiments

MDA-MB-231 cells were serum starved overnight and stimulated with EGF (100 ng/ml, 37 °C) for the indicated times. Co-immunoprecipitation experiments were described previously [24]. Briefly, cells were lysed into ice-cold TGH buffer (pH 7.3, 50 mM HEPES, 50 mM NaCl, 5 mM EDTA, 10% glycerol, 1% triton and protease inhibitors) and β1-integrin, talin, paxillin, ARF1 or EGFR were immunoprecipitated using the anti-β1-integrin, anti-talin, anti-paxillin, anti-ARF1 or anti-EGFR antibodies. Interacting proteins were assessed by Western blot analysis.

2.6. GTPase activation assay

MDA-MB-231 cells transfected with scrambled siRNA or an siRNA targeting FAK (50 nM) were stimulated with EGF (10 ng/ml) for the indicated times and then washed with TBS and lysed in 200 μl of ice-cold MLB lysis buffer (pH 7.5, 25 mM HEPES, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% nonidet P-40, 0.3 mg/ml PMSF, 1.0 mM Na_3VO_4 and protease inhibitors). Samples were vortexed for 10 s and spun for 10 min at 10,000 g. GST-GGA3 coupled to glutathione sepharose 4B were added to each tube and samples were rotated at 4 °C for 45 min. Beads were washed and proteins were eluted in 25 μl SDS sample buffer by heating at 65 °C for 15 min. Detection of ARF1-GTP was performed by immunoblot analysis using a specific anti-ARF1 antibody. A similar methodology was used for the detection of activated ARF1 in conditions where cells were treated with the ARF1 biochemical inhibitor LM11. For those experiments, MDA-MB-231 cells were simply first treated with vehicle (dimethyl sulfoxide (DMSO) 1%) or LM11 (indicated concentration) before being stimulated.

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