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Original article

EBP50 promotes focal adhesion turnover and vascular smooth muscle cells migration ☆

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ABSTRACT

The ezrin–radixin–moesin-binding phosphoprotein 50 (EBP50) is a PDZ-containing scaffolding protein that regulates a variety of physiological functions. In the vasculature, EBP50 promotes neointima formation following arterial injury. In this study the role of EBP50 on vascular smooth muscle cell (VSMC) migration was characterized. The spreading and motility of primary VSMC isolated from EBP50 knockout (KO) mice were significantly reduced compared to wild-type (WT) cells. EBP50-null VSMC had fewer and larger focal adhesions than wild-type cells. Assembly and disassembly of focal adhesion—assessed by live-cell total internal reflection fluorescence imaging—in response to epidermal growth factor (EGF) were significantly reduced in KO cells. Immunoprecipitation experiments showed that EBP50 interacts with EGF receptor via the PDZ2 domain and with focal adhesion kinase (FAK) via the C-terminal ERM domain. EBP50 promoted the formation of a complex containing both EGF receptor and FAK. Phosphorylation of Tyr-925 of FAK in response to EGF was significantly reduced in KO cell compared to WT cells. The residence time of FAK in focal adhesions—determined by fluorescence recovery after photobleaching—was increased in WT cells. Collectively, these studies indicate that EBP50, by scaffolding EGF receptor and FAK, facilitates activation of FAK, focal adhesion turnover, and migration of VSMC.

1. Introduction

Cell migration is a fundamental cellular process that is important during morphogenesis and tissue regeneration and repair [1,2]. However, unregulated cell migration is a major factor in tumor progression and metastasis [3,4], and in a number of vascular pathologies [5,6]. Focal adhesions (FAs) are the cellular microdomains that mediate cell migration [7]. The coordinated formation of FAs at the leading edge and the disassembly of FAs at the trailing edge of a migrating cell provide the directional force for movement [8,9]. Up to 100 signaling and structural molecules within FAs regulate this dynamic turnover in response to biochemical signals originating from membrane receptors [10]. Among these, the non-receptor tyrosine kinase focal adhesion kinase (FAK) plays a central role in regulating FA dynamic and cell motility [11,12]. Numerous studies using FAK-null cells and overexpression of wild-type and dominant-negative FAK have demonstrated the essential role of this protein in cell migration [13–15]. In addition, evidence for the importance of tyrosine phosphorylation of FAK in regulating its function has emerged. Tyr-397 is an essential site that is auto-phosphorylated upon integrin engagement [16]. Following Tyr-397 phosphorylation, src family kinases phosphorylate other residues (407, 576, 577, 861 and 925) in a cell-type dependent manner [17,18]. In particular, Tyr-925 phosphorylation has been shown to promote FAK residence time in FAs, FA turnover and cell migration in fibroblasts [19].

The ezrin–radixin–moesin-binding phosphoprotein 50 (EBP50), also known as Na^+/H^+ exchanger regulatory factor 1 (NHERF1), is a

Abbreviations: EBP50, ezrin–radixin–moesin-binding Phosphoprotein 50; EGF, epidermal growth factor; EGFR, EGF receptor; ERM, ezrin–radixin–moesin; FA, focal adhesion; FAK, focal adhesion kinase; FBS, fetal bovine serum; GFP, green fluorescent protein; KO, knockout; PDZ, postsynaptic density 95/disks large/zona occludens; VSMC, vascular smooth muscle cell; WT, wild type; YFP, yellow fluorescent protein.

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PDZ domain-containing scaffolding protein [20]. EBP50 was originally identified as a critical regulator of Na^+/H^+ exchanger 3 in the kidney [21]. In addition to the well-established regulation of ion homeostasis in the kidney and intestine [22–24], EBP50 exerts important actions on liver biology [25,26] as well as type-specific contributions to growth and metastatic potential of various cancers, including breast, liver, intestine and brain tumors [27–30].

Recently, significant actions of EBP50 in the vasculature have emerged. EBP50 depletion potentiates the contractile response of mesenteric arteries to noradrenaline [31]. Our studies showed that EBP50 expression increases in arteries following endoluminal denudation and contributes to neointima formation by positively regulating vascular smooth muscle cell proliferation [32,33]. However, the remarkable inhibition of neointimal hyperplasia in EBP50-null mice suggests that other cellular responses may be affected by EBP50. Of these, VSMC migration is of particular interest because the acquisition of motility is one of the principal phenotypic changes associated with vascular remodeling and the response of VSMC to growth factors and cytokines [5].

Because of the fundamental role of FAs on cell motility and the importance of FAK function in regulating FA dynamics we sought to determine the effect of EBP50 on these processes. To this end we used a combination of biophysical and biochemical approaches to establish the effect of EBP50 on FAK activity and growth factor-induced VSMC migration.

2. Materials and methods

2.1. Plasmids and mutagenesis

The plasmid encoding N-terminal Flag-human EBP50 was a gift from Dr. Peter Friedman (University of Pittsburgh). The mutants S1, S2, ΔERM and S1/S2 EBP50 mutant constructs were made from Flag-EBP50 by using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Mutagenic primers were designed based on human EBP50 sequence (S1 mutant, 5′-AGGGTCCGAAC GGCGCCGGCGCCCACCTGCACGGGG-3′; S2 mutant, 5′-GAAGAAGGG CCCCAGTGGCGCTGGCGCCAACCTGCACAGCGACAAGTC-3′; delta ERM, 5′-GACTTCAACATCTCCATGAGCGCGGACGCA-3′). S1/S2 mutagenesis was done by two consecutive mutagenic reactions using S1 and S2 mutagenic primers. DNA sequences were confirmed by sequence analysis (GeneWiz).

2.2. Primary VSMC culture and transfection

All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Primary VSMCs were isolated from abdominal aortic explants from wild type (WT) C57BL/6 and EBP50 knockout (KO) littermate mice and cultured in Dulbecco's modified eagle media (DMEM) containing 10% fetal bovine serum (FBS) in 5% CO_2 at 37 °C. All experiments were performed with cells between passages 3 and 15. CHO cells were cultured in Ham's F-12 medium supplemented with 10% FBS. For transient EBP50 expression, EBP50 plasmid was introduced in primary VSMC by electroporation using an AMAXA electroporator and the Basic Nucleofect kit (Lonza). For GFP-tagged FAK expression, primary VSMCs were infected with an adenovirus encoding GFP-FAK (a generous gift from Dr. Harold Singer, Albany Medical College, NY). Cells were incubated with adenovirus in serum free media for 1 h and incubated with 10% FBS-supplemented media overnight. Experiments were performed at 24 h after infection. CHO cells were transfected using Lipofectamine 2000 (Invitrogen). Transfections with siEBP50 and infections with lentiviral shEBP50 were performed as described previously [33]. Cells were used after 3 days of infection.

2.3. Cell migration assays

Cell migration was analyzed by a scratch wound assay. Cells were grown to confluence in 12-well plates. A scratch wound in the monolayer was performed by dragging a pipette tip across the layer. Detached cells were washed away with PBS. The remaining cells were culture in 10% FBS DMEM or treated as described after 24 h in 0.1% FBS. The closure of the wound over time was monitored by light microscopy (Olympus IX71). To measure the velocity of cell migration, VSMCs were monitored for up to 24 h on a Nikon TE2000 inverted microscope equipped with a motorized stage and a LiveCell stage incubator system (Pathology Devices, Inc.) that provides temperature, humidity and CO_2 control. Images were captured every 20 min using a ×10 objective and a Nikon DS-Fi1 CCD camera controlled by NIS Elements software (Nikon). Moving distances of single cells over time were measured using Image J software (National Institutes of Health). Boyden chamber migration assays were performed using 24-well transwell chambers (BD bioCoat) with a pore size of 8 μm. The upper compartment was filled with 300 μl of a cell suspension containing 5×10^4 cells. Bottom wells were filled with 500 μl of basal medium (DMEM containing 0.5% FBS) without or with growth factors (EGF 10 ng/ml or PDGF 10 ng/ml). Cells were then allowed to migrate for 5 h at 37 °C. Cells were removed from the upper surface with cotton swabs, and the filters were washed in PBS. Cells were fixed in 4% paraformaldehyde in PBS, and stained with 0.1% crystal violet. Images were acquired on an Olympus IX71 microscope using a 20× objective.

2.4. Immunofluorescence analysis

Cells on glass coverslips were fixed with 4% paraformaldehyde and incubated with blocking buffer containing 5% goat serum and 0.2% Nonidet P-40 (NP-40) in PBS. Primary rabbit anti-FAK, anti-paxillin (both Santa Cruz, 1:500), anti-phospho paxillin, vinculin (both Sigma, 1:500) antibodies or phalloidin-red (Cell signaling, 1:2000) were applied in the same buffer overnight at 4 °C. Coverslips were washed with PBS, incubated with Alexa546-conjugated anti-rabbit secondary antibody (1:1000, Molecular Probes) and 4′,6-diamidino-2-phenylindole (DAPI, 0.1 μg/ml; Sigma) for 2 h and washed again. Coverslips were mounted for immunofluorescence microscopy and analyzed with an Olympus Fluoview confocal laser-scanning microscope with a ×63 oil immersion objective. The fraction of phospho-paxillin that co-localizes with vinculin (Mander's coefficient) was calculated with the JACoP plug-in for Image J [34].

2.5. Live cell imaging

Total internal reflection fluorescence (TIRF) images were acquired using a Nikon Ti-TIRF microscope equipped with 60× Oil TIRF objective (NA = 1.49). VSMCs infected with adenovirus encoding GFP-FAK were imaged every minute for 60 min. The fluorescence intensity, the number and the size of focal adhesions of each image were determined with Image J software (National Institutes of Health). Assembly of FAs was calculated by subtracting the fluorescence image at 30 min from the image at 10 min followed by normalization to the total fluorescence. Conversely, disassembly of FAs was calculated by subtracting the fluorescence image at 10 min from the image at 30 min followed by normalization to the total fluorescence.

Fluorescence recovery after photobleaching (FRAP) measurements were performed on an Olympus Fluoview confocal laser-scanning microscope with a 100× water immersion objective. Single focal adhesions were photobleached for 2 s at 100% of the 488- and 545-nm laser lines. Fluorescence recovery was recorded every 3 s for 2 min and corrected to the fluorescence of an unbleached region. Fluorescence intensity at each time point was then normalized to the prebleached intensity of the FA.