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Human enhancer of filamentation 1-induced colorectal cancer cell migration: Role of serine phosphorylation and interaction with the breast cancer anti-estrogen resistance 3 protein

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ABSTRACT

Human enhancer of filamentation 1 (HEF1) is a member of the p130Cas family of docking proteins involved in integrin-mediated cytoskeleton reorganization associated with cell migration. Elevated expression of HEF1 promotes invasion and metastasis in multiple cancer cell types. To date, little is known on its role in CRC tumor progression. HEF1 is phosphorylated on several Ser/Thr residues but the effects of these post-translational modifications on the functions of HEF1 are poorly understood. In this manuscript, we investigated the role of HEF1 in migration of colorectal adeno-carcinoma cells. First, we showed that overexpression of HEF1 in colo-carcinoma cell line HCT116 increases cell migration. Moreover, in these cells, HEF1 increases Src-mediated phosphorylation of FAK on Tyr-861 and 925. We then showed that HEF1 mutation on Ser-369 enhances HEF1-induced migration and FAK phosphorylation as a result of protein stabilization. We also, for the first time characterized a functional mutation of HEF1 on Arg-367 which mimics the effect of Ser-369 to Ala mutation. Finally through mass spectrometry experiments, we identified BCAR3 as an essential interactor and mediator of HEF1-induced migration. We demonstrated that single amino acid mutations that prevent formation of the HEF1-BCAR3 complex impair HEF1-mediated migration. Therefore, amino-acid substitutions that impede Ser-369 phosphorylation stabilize HEF1 which increases the migration of CRC cells and this latter effect requires the interaction of HEF1 with the NSP family adaptor protein BCAR3. Collectively, these data reveal the importance of HEF1 expression level in cancer cell motility and then support the utilization of HEF1 as a biomarker of tumor progression.

1. Introduction

HEF1, also known as Nedd9 or Cas-L, is one of the proteins that have been associated with tumor progression. Indeed, HEF1 up-regulation has been linked to enhanced invasion and metastasis in different cancer types including breast, glioblastoma, head and neck squamous cell carcinomas, lung and melanoma (Izumchenko et al., 2009; Ji et al., 2007; Kim et al., 2006; Kong et al., 2011; Lucas et al., 2010; Natarajan et al., 2006). HEF1 involvement in tumor development depends on cancer cell type where its contribution includes tumor growth (Li et al., 2011; Little et al., 2013), metastasis process, epithelial-mesenchymal transition (Kong et al., 2011; Tikhmyanova and Golemis, 2011), mesenchymal movement (Sanz-Moreno et al., 2008) and invasion (Fashena et al., 2002). Recently, Li et al. reported an increased HEF1 expression in colorectal cancer as a target of Wnt signaling promoting cell migration (Li et al., 2011). Although most studies described a positive effect of HEF1 on motility, HEF1 can negatively regulate MCF10A migration and its down-regulation is part of a signature for breast cancer metastasis (Minn et al., 2005; Simpson et al., 2008). These apparent controversial data highlight the complexity of HEF1 involvement in tumor progression.

Abbreviations: BCAR3, breast cancer anti-estrogen resistance 3; Cas, Crk-associated substrate; CHAT-H, Cas/HEF1-associated signal transducer-hematopoietic; CHX, cycloheximide; CRC, colorectal cancer; FAK, focal adhesion kinase; GEF, guanine nucleotide-exchange factor; HEF1, human enhancer of filamentation-1; HSC70, heat shock cognate 70; IHC, immunohistochemistry; MMP, matrix metalloproteinase; Nd, nocodazole; Nedd9, neural precursor cell expressed, developmentally down-regulated 9; NSP, novel SH2-containing proteins; OA, okadaic acid; PGE2, prostaglandin E2; PP2A, protein phosphatase 2A; SH2, Src homology domain-2.

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HEF1 belongs to the p130Cas family of proteins. It is a multi-domain scaffolding protein participating to the integrin dependent signaling in a complex with the tyrosine kinases FAK and Src which in turn, regulate various downstream signaling pathways. FAK constitutes a key component of the migration machinery and is primarily activated through an auto-phosphorylation on Tyr-397 residue then undergoes additional phosphorylations on Tyr-576/Tyr-577, Tyr-861 and Tyr-925 in a Src family kinases dependent manner. This pathway controls downstream cellular processes like adhesion, migration and/or proliferation (Abu-Ghazaleh et al., 2001; Deramaudt et al., 2011; Mitra and Schlaepfer, 2006). In addition to tyrosine kinases, HEF1 can form a complex with tyrosine phosphatases, adaptors, ubiquitin ligases, or GEF domain proteins. NSP (Novel Sh2 containing Proteins) belong to this latter family of proteins whose GEF activity has not been formally established but their interaction with p130Cas family indirectly regulates GTPases and contributes to cell motility and adhesion remodeling (Wallez et al., 2012). These proteins have been linked to increased cell migration in various cancers (Guerrero et al., 2012; Wallez et al., 2012).

Besides the well-documented correlation between tumor progression and increased HEF1 expression, much less is known about the role of HEF1 post-translational modifications, especially phosphorylations, on its properties. HEF1 possesses a substrate domain containing several YXXP motifs and a serine-rich region; both constituting potential phosphorylation sites. As in a vast majority of proteins, these phosphorylations presumably fundamentally modify HEF1 functions. Indeed, HEF1 is a FAK and Src substrate, its interacting properties being regulated in part by tyrosine phosphorylations (O'Neill et al., 2000, Singh et al., 2007). Furthermore, HEF1 undergoes Ser/Thr phosphorylations. As a matter of fact, on Western blot, HEF1 appears as two species of different electrophoretic mobility referred to as p105 and p115 (Zheng and McKeown-Longo, 2002, 2006). To date two Ser/Thr phosphorylation sites have been described. HEF1 interacts with and activates Aurora A kinase which in turn phosphorylates HEF1 on Ser-296 (Pugacheva and Golemis, 2005). In addition, we have previously identified another phosphorylation on Ser-369 and have shown that it accounts for the p115 shift and targets HEF1 to degradation by the proteasome (Hivert et al., 2009). Little data are available on regulation of these phosphorylations. Phosphorylation of HEF1 on Ser/Thr residues and appearance of the p115 isoform have been described as a result of cell adhesion; furthermore, p115 is the species preferentially targeted to proteasomal degradation (Nourry et al., 2004, Zheng and McKeown-Longo, 2006). HEF1 Ser/Thr dephosphorylation seems to depend on PP2A phosphatase (Zheng and McKeown-Longo, 2006). Finally, recently, HEF1 mutations were reported in tumor samples in several databases (http://cancer.sanger.ac.uk/; http://www.cbioportal.org/). However, to date the functional significance of these mutations has not been identified.

In this study, using cancer cell line models, we investigated the mechanisms by which HEF1 is regulated and increases cell migration.

2. Materials and methods

2.1. Cell culture

Human colorectal cancer cell lines (HCT116, Caco-2, SW480, Isreco-1 and Colo-205) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). All cell lines except HCT116 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Fisher Scientific, Illkirch, FR) supplemented with antibiotics (50 μg/ml penicillin, 50 μg/ml streptomycin), 1 mM sodium pyruvate and 10% fetal calf serum (FCS) at 37 °C with 5% CO_2 atmosphere. HCT116 cell line was cultured in McCoy's 5 A medium (Fisher Scientific, Illkirch, FR) with antibiotics, sodium pyruvate and 10% FCS as mentioned above.

2.2. Reagents and antibodies

Okadaic acid and the Src inhibitor (PP2) were purchased from Calbiochem (VWR, Strasbourg, FR) and were added to the culture medium at final concentration of 125 nM and 2 μM respectively. Cycloheximide (CHX) and Nocodazole (Nd) were purchased from Sigma–Aldrich (St Louis, MO, USA) and were used at final concentration of 140 μM and 300 nM respectively. Anti-HEF1 (2G9) monoclonal antibody was from ImmuQuest (Antibodies-online, Aachen, Germany) Anti-Flag (M2) monoclonal antibody, anti-BCAR3, anti-NSP1 and puromycin were from Sigma–Aldrich (St Louis, MO, USA). Anti-V5 antibody was from ABD Serotec (Dusseldorf, Germany). Anti-Phospho-Ser-369-HEF1 polyclonal antibody was generated as previously described (Hivert, Pierre, 2009). Anti-HSC70 monoclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-FAK and anti-Phospho-Tyr FAK antibodies were from Cell Signaling Technology (Ozyme, Saint Quentin en Yvelines, FR). Doxycycline was from Enzo Life sciences (Lyon, FR).

2.3. Expression of exogenous proteins

For inducible overexpression of Flag-HEF1, the plasmid pTRIPZ-Flag-HEF1 was generated as follows: the complete coding sequence of HEF1 was cut with *XmaI*/*XhoI* restriction enzymes from a pCMV-Flag-HEF1 vector previously constructed (Hivert et al., 2009), and then cloned in the pTRIPZ lentiviral vector (Open BioSystems, Huntsville, Al, USA) downstream of a promoter regulated by a tetracycline responsive element. A stop codon linker was synthesized using following oligonucleotides: 5′-TCGAGTGACCGGTACCGGCGCGCCTACGTATG-3′ (sense), 5′-AATTCATACGTAGGCGCGCCGGTACCGGTCACT-3′ (antisense). When hybridized, these oligonuceotides gave a double stranded fragment with *XhoI* and *EcoRI* ends that was inserted into pTRIPZ vector, downstream of HEF1 sequence, between *XhoI* and *EcoRI* restriction sites. pTRIPZ-Flag-HEF1 S296A, pTRIPZ-Flag-HEF1 S369A, pTRIPZ-Flag-HEF1 R367Q and pTRIPZ-Flag-HEF1 L751D were generated using oligonucleotide-directed PCR mutagenesis to create a Ser to Ala, an Arg to Gln or a Leu to Asp replacement at amino-acids 296, 369, 367 and 751 respectively. Cells infected with the different Flag-HEF1 containing plasmids or the related empty vector were selected with puromycin at 2 μg/ml. To induce Flag-HEF1 expression, cells were treated with 250 ng/ml doxycycline (unless stated differently in the figure legends) for 48 h prior to experiments. For BCAR3 stable overexpression, BCAR3 cDNA was first mutated on the sequence targeted by the BCAR3 shRNA using the oligonucleotides: 5′-GCTATGGGGCAGCAAAGGAGCCCAGGTGAACCAGACAGAGAGATATGAG-3′ (sense) and 5′-CTCATATCTCTCTGTCTGGTTCACCTGGGCTCCTTTGCTGCCCCATAGC-3′ (antisense) and inserted, through the Gateway® technology, from pDONR-BCAR3 (Thermo Scientific, Rockford, IL, USA) into pLX-302 lentiviral vector (Addgene, Cambridge, MA, USA). pLX-302-BCAR3 R748A vector was generated from pLX-302-BCAR3 wt vector by oligonucleotide-directed PCR mutagenesis. Cells infected with BCAR3 related plasmids were selected with blasticidin at 10 μg/ml (Cayla, Toulouse, FR). Correct sequence of all plasmids was confirmed by DNA sequencing.

2.4. shRNA-mediated knockdown of HEF1, BCAR3 and NSP1

To turn off the expression of endogenous HEF1, BCAR3 and NSP1 proteins, lentiviral vectors pLKO-shHEF1, pGIPZ-shBCAR3