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DOCK1 inhibition suppresses cancer cell invasion and macropinocytosis induced by self-activating Rac1^{P29S} mutation

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ABSTRACT

Rac1 is a member of the Rho family of small GTPases that regulates cytoskeletal reorganization, membrane polarization, cell migration and proliferation. Recently, a self-activating mutation of Rac1, Rac1^{P29S}, has been identified as a recurrent somatic mutation frequently found in sun-exposed melanomas, which possesses increased inherent GDP/GTP exchange activity and cell transforming ability. However, the role of cellular Rac1-interacting proteins in the transforming potential of Rac1^{P29S} remains unclear. We found that the catalytic domain of DOCK1, a Rac-specific guanine nucleotide exchange factor (GEF) implicated in malignancy of a variety of cancers, can greatly accelerate the GDP/GTP exchange of Rac1^{P29S}. Enforced expression of Rac1^{P29S} induced matrix invasion and macropinocytosis in wild-type (WT) mouse embryonic fibroblasts (MEFs), but not in DOCK1-deficient MEFs. Consistently, a selective inhibitor of DOCK1 that blocks its GEF function suppressed the invasion and macropinocytosis in WT MEFs expressing Rac1^{P29S}. Human melanoma IGR-1 and breast cancer MDA-MB-157 cells harbor Rac1^{P29S} mutation and express DOCK1 endogenously. Genetic inactivation and pharmacological inhibition of DOCK1 suppressed their invasion and macropinocytosis. Taken together, these results indicate that DOCK1 is a critical regulator of the malignant phenotypes induced by Rac1^{P29S}, and suggest that targeting DOCK1 might be an effective approach to treat cancers associated with Rac1^{P29S} mutation.

1. Introduction

Rac1, which was originally discovered as Ras-related C3 botulinum toxin substrate 1 [1], is a member of the Rho family of small guanosine triphosphatases (GTPases) and functions as a molecular switch cycling between a GDP-bound inactive state and a GTP-bound active state [2,3]. Conversions to the active state and to the inactive state occur via GDP/GTP exchange and hydrolysis of GTP to GDP, respectively. Normally, Rac1 exists in the cells as a GDP-bound inactive state due to its marginal intrinsic GDP/GTP exchange activity and by the functions of GTPase-activating proteins (GAPs). Thus, activation of Rac1 requires the function of proteins called guanine nucleotide exchange factors (GEFs) that facilitate the nucleotide exchange. Once activated, Rac1 associates with a variety of downstream effectors and controls fundamental cellular processes including morphogenesis, migration, and proliferation through the regulation of cytoskeletal reorganization, membrane dynamics, and gene expression [2,3]. Aberrant expression and dysregulation of the activity of Rac1 have been linked to a variety of human cancers [4,5]. In cancer cells, activation of Rac1 leads to the

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formation of highly dynamic, actin-rich membrane structures such as invadopodia and circular dorsal ruffles that are implicated in three-dimensional migration, matrix invasion, and macropinocytosis [6–8]. Three-dimensional migration and matrix invasion are the basal mechanism of metastasis. Macropinocytosis is an endocytic process that internalizes a large portion of plasma membrane as well as extracellular fluid and its contents [8,9]. In Ras-transformed cells, Rac1-mediated macropinocytosis is exploited to feed on extracellular protein and supply amino acids for their sustained survival [10,11]. Thus, the invasion and macropinocytic activities facilitated by Rac1 contribute to tumorigenic and metastatic phenotypes of cancer cells.

Besides overexpression and alternative splicing [4,5], cancer-associated mutations of Rac1 have not been found frequently until recent, large genomic sequencing studies showing several gain-of-function mutations of Rac1 in human cancers [12–14]. Among the mutations, a hot spot C > T mutation in the RAC1 gene that changes proline at codon 29 to serine ($Rac1^{P29S}$) is a recurrent somatic mutation found in 9% of sun-exposed malignant melanomas, the third most frequent activating mutation in such melanomas after those of BRAF and NRAS [12]. $Rac1^{P29S}$ is shown biochemically to be self-activated due to substantially increased inherent GDP/GTP exchange [14,15]. Expression of $Rac1^{P29S}$ induces membrane ruffles, cell migration, proliferation, anchorage-independent growth, and gene expression in various cells [13–15]. So far, however, the role of cellular Rac1-interacting proteins in the transforming potential of $Rac1^{P29S}$ remains to be explored.

Two distinct families of Rac GEFs, the Dbl family and DOCK family, are currently known [16,17]. The DOCK family proteins are evolutionarily conserved, Rac/Cdc42 specific GEFs containing the lipid-binding DHR-1 (DOCK homology region-1) and the catalytic DHR-2 domains [17]. DOCK1 functions as a Rac specific GEF and regulates phagocytosis, myoblast fusion, and cell migration [17]. DOCK1 is critical for circular dorsal ruffle formation [18]. Aberrant expression and activity of DOCK1 is associated with malignant phenotypes in a variety of cancers [19–22]. Several lines of evidence indicate that DOCK1 regulates invasion and metastasis by acting downstream of receptor tyrosine kinases in glioblastoma and breast cancer cells [20–22]. Moreover, our recent study revealed that DOCK1 is a critical regulator of Rac activation that promotes invasion and macropinocytosis in Ras-driven cancer cells [23]. In this study, we found that DOCK1 can promote the GDP/GTP exchange of $Rac1^{P29S}$, and is essential for $Rac1^{P29S}$-induced invasion and macropinocytosis in mouse embryonic fibroblasts. Moreover, we show by genetic inactivation and pharmacological inhibition that DOCK1 inhibition can suppress the invasion and macropinocytosis in human cancer cells harboring $Rac1^{P29S}$ mutation.

2. Materials and methods

2.1. In vitro nucleotide exchange assays

Recombinant His-SUMO-tagged DOCK1 DHR-2 domain and GST-fused Rac1 proteins were prepared as described previously [23,24]. The assays consisted of GST-Rac1 (10 μM), His-SUMO-DOCK1 DHR-2 domain (0.05 μM), and Bodipy-FL-GTP (2.4 μM; G12411, Invitrogen) in the reaction buffer: 20 mM MES-NaOH, 150 mM NaCl, 1 mM $MgCl_2$, and 20 μM GDP, pH 7.0. GST-Rac1 was loaded with GDP by incubating with the reaction buffer on ice for 30 min, then mixed with Bodipy-FL-GTP and allowed to equilibrate at 30 °C for 2 min. The reaction was initiated by adding His-SUMO-DOCK1 DHR-2 domain (50 μl) to the GDP-loaded GST-Rac1/Bodipy-FL-GTP mixture (100 μl) in a final volume of 150 μl and incubating at 30 °C. The change in the fluorescence (Excitation: 488 nm/Emission: 514 nm) was recorded for 15 min with 15-s intervals using the Perkin Elmer EnSpire multimodal plate reader. Data were fitted with the curve fitting function of GraphPad Prism (GraphPad Software) to calculate the initial slope during the first 10 s (RFU/s: relative fluorescent unit per second).

2.2. Cell preparation and culture

Wild-type (WT) and DOCK1-deficient ($DOCK1^{-/-}$) MEFs were immortalized and infected with recombinant retroviruses as described previously [23], using retrovirus vectors pMX-IRES-GFP encoding N-terminally HA-tagged $Rac1^{WT}$ and $Rac1^{P29S}$. Clones with comparable expression of HA-tagged Rac1 proteins were selected for functional analyses. A human skin malignant melanoma cell line IGR-1 was purchased from Cell Lines Service (#300219). DOCK1-deficient (DOCK1-KO) IGR-1 was generated by the CRISPR/Cas9 system in combination with gene targeting by homologous recombination as previously described [23]. DOCK1-KO clones (#1 and #2) with different target guide sequences (target 1 and 2 [23], respectively) were selected. A human breast cancer cell line MDA-MB-157 was purchased from American Type Culture Collection. Cells were cultured in DMEM medium (Wako Pure Chemical Industries) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Nichirei Bioscience), 100 U/ml penicillin (Life Technologies), and 100 μg/ml streptomycin (Life Technologies).

2.3. Immunoblotting

Total cell lysates were prepared as described previously [23], and analyzed by immunoblotting with the following primary antibodies: rabbit anti-DOCK1 (#C4C12, Cell Signaling Technology, 1:1000 dilution), mouse anti-Rac1 (#23A8, Millipore, 1:3000), rabbit anti-HA-tag (#C29F4, Cell Signaling Technology, 1:2000 dilution), and anti-β-actin (I-19, Santa Cruz, 1:1000 dilution), and secondary antibodies conjugated with horse radish peroxidase (HRP): anti-rabbit immunoglobulin (IgG) (sc-2004, Santa Cruz, 1:2000 dilution), anti-mouse IgG (sc-2005, Santa Cruz, 1:2000 dilution), and anti-goat IgG (sc-2020, Santa Cruz, 1:2000 dilution). Twenty μg protein of total cell lysate per lane was used for Rac1 blot, and 50 μg protein for the others.

2.4. Invasion assays

Matrix invasion activity was measured by using the BioCoat matrigel invasion chambers (Corning, #354480). Briefly, cells (2.5×10^4 cells for MEFs, and 5×10^4 cells for MDA-MB-157) were suspended in serum-free medium (300 μl) and loaded onto the upper chamber with an 8 μm pore size membrane coated with a thin layer of Matrigel basement membrane matrix, which was placed in a 24-well plate containing DMEM supplemented with 5% FBS (MEFs) or 20% FBS (MDA-MB-157) (500 μl). When applied, cells were incubated with mediums supplemented with TBOPP (final concentrations of 6.25–12.5 μM) or DMSO (vehicle control, final 0.2%). After incubation for 22 h (MEFs) or 48 h (MDA-MB-157) at 37 °C, cells on the bottom side of the upper chamber membrane (invaded cells) were stained with a modified Giemsa method using the Diff-Quick kit (Sysmex), and then analyzed by an Axio Lab.A1 light microscope (Carl Zeiss). For each sample, the average number of invaded cells from four separate fields was determined.

2.5. Macropinocytosis assays

Macropinocytic activity was assessed by measuring the uptake of fluorescently labeled dextran [23]. Briefly, cells were seeded on fibronectin-coated glass bottom dishes (Matsunami Glass) for

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