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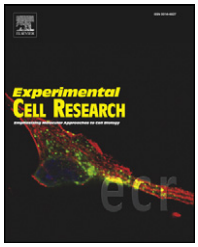

Research Article

Ephexin4 and EphA2 mediate resistance to anoikis through RhoG and phosphatidylinositol 3-kinase

*Kohei Harada, Nao Hiramoto-Yamaki, Manabu Negishi, Hironori Katoh**

Laboratory of Molecular Neurobiology, Graduate School of Biostudies, Kyoto University, Yoshidakonoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

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ABSTRACT

Disruption of cell–extracellular matrix interaction causes epithelial cells to undergo apoptosis called anoikis, and resistance to anoikis has been suggested to be a critical step for cancer cells to metastasize. EphA2 is frequently overexpressed in a variety of human cancers, and recent studies have found that overexpression of EphA2 contributes to malignant cellular behavior, including resistance to anoikis, in several different types of cancer cells. Here we show that Ephexin4, a guanine nucleotide exchange factor for the small GTPase RhoG that interacts with EphA2, plays an important role in the regulation of anoikis. Knockdown of Ephexin4 promoted anoikis in HeLa cells, and experiments using a knockdown-rescue approach showed that activation of RhoG, phosphatidylinositol 3-kinase (PI3K), and Akt was required for the Ephexin4-mediated suppression of anoikis. Indeed, Ephexin4 knockdown caused a decrease in RhoG activity and Akt phosphorylation in HeLa cells cultured in suspension. In addition, Ephexin4 was involved in the EphA2-mediated suppression of anoikis. Taken together, these results suggest that Ephexin4 mediates resistance to anoikis through activation of RhoG and PI3K downstream of EphA2.

Introduction

In normal epithelial cells, cell–extracellular matrix interaction is required for cell survival and proliferation, and when detached from the extracellular matrix, they undergo apoptosis, a phenomenon called anoikis [1–4]. In contrast, tumor cells that acquire malignant potential are capable of evading anoikis and surviving after detachment from their primary site. The acquisition of anoikis resistance is therefore regarded as a critical step for tumor cells to metastasize [5–7]. Numerous signaling molecules including kinases and small G proteins have been implicated in the regulation of anoikis. One important and well-studied pathway involves phosphatidylinositol 3-kinase (PI3K) and its downstream target Akt, and a number of Akt substrates that are important for the regulation of cell survival have been identified [8–11]. In addition, a number of signaling pathways including integrins and Bcl2 family proteins have been implicated in anoikis [2–4].

It is already well known that members of the Rho family small GTPases are key regulators of the actin cytoskeleton in diverse cellular functions [12–14]. They also play important roles in cell proliferation and survival [15–17]. Activation of Rho family GTPases requires GDP–GTP exchange catalyzed by various guanine nucleotide exchange factors (GEFs). The major class of GEFs is the Dbl family GEFs that contain the Dbl homology–pleckstrin homology (DH–PH) tandem domain and mediate the GDP–GTP exchange through the DH domain. The second class of GEFs for Rho family GTPases is the Dock family GEFs that have no DH–PH tandem domain but contain a conserved domain that directly

* *Corresponding author.* Fax: +81 75 753 7688.
E-mail address: hirokato@pharm.kyoto-u.ac.jp (H. Katoh).

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interacts with Rho GTPase and mediates its GDP–GTP exchange [18,19].

RhoG is a member of Rho family small GTPases that is a key upstream regulator of another Rho family member Rac to induce diverse cellular functions, including promotion of cell migration, neurite outgrowth in neuronal cells, and stimulation of phagocytosis [20–23]. ELMO, an effector of RhoG, forms a complex with Dock180 or Dock4, which serves as a functional GEF for Rac in intact cells, and the interaction of RhoG with ELMO induces translocation of the ELMO–Dock180 or ELMO–Dock4 complex from the cytoplasm to the plasma membrane and activates Rac [24–26]. On the other hand, RhoG regulates cell proliferation and survival via PI3K independently of its ability to activate Rac [27–29]. RhoG interacts with the PI3K regulatory subunit, p85α, and activates the PI3K-Akt signaling pathway to promote resistance to anoikis in HeLa cells [28]. However, the upstream regulators of RhoG activity in the regulation of anoikis still remain obscure.

The Eph receptors are the largest family of receptor tyrosine kinases. Their ligands, ephrins, are membrane-anchored proteins which are divided into two subclasses: class A and class B ephrins, and there are also two classes of Eph receptors, EphA and EphB, based on homology and binding affinity for class A and class B ephrins. Ephrin/Eph receptor signaling pathways have many important functions during development and in tissue homeostasis, and many studies have shown that dysregulation of ephrin/Eph receptor signaling contributes to cancer progression [30–32]. Eph receptors contain a protein kinase domain and a sterile-α-motif (SAM) domain in the cytoplasmic region and bind to diverse signaling effectors that regulate the activities of kinases and small GTPases. Among them, Ephexin was identified as a subfamily of Dbl types Rho GEFs that interacts directly with Eph receptors [33,34]. At least five members of the Ephexin subfamily (Ephexin1–5) have been reported, and Ephexin1 (ARHGEF27/NGEF), Ephexin2 (ARHGEF19/WGEF), Ephexin3 (ARHGEF5/TIM1), and Ephexin5 (ARHGEF15/Vsm-RhoGEF) activate RhoA [33–37]. It has been reported that Ephexin1 regulates axon guidance and spine morphogenesis through the interaction with EphA4 [34,38]. Ephexin5/Vsm-RhoGEF also interacts with EphA4 to regulate vascular smooth muscle cell contractility [35], and a recent study has shown its interaction with EphB2 to regulate excitatory synapse formation in hippocampal neurons [39]. On the other hand, Ephexin2 is involved in the planar cell polarity signaling pathway to regulate epidermal wound repair [40]. We recently reported that Ephexin4 interacts with EphA2 and activates RhoG [41]. EphA2 is frequently overexpressed in a variety of human cancers, and recent studies have found that overexpression of EphA2 contributes to malignant cellular behavior, including resistance to anoikis, in several different types of cancer cells, independently of the ligand ephrin stimulation. Conversely, stimulation of EphA2 with its ligand ephrinA1 in cancer cells inhibits cell proliferation and migration [42–46]. In breast cancer cells, EphA2 interacts with Ephexin4 and induces ligand-independent promotion of cell migration and invasion through the Ephexin4-mediated activation of RhoG [41]. However, the molecular mechanisms underlying other malignant cellular behavior by EphA2 remain poorly understood. In this study, we have investigated the role of Ephexin4 in the regulation of anoikis. We show that Ephexin4 is involved in the EphA2-mediated suppression of anoikis in HeLa cells through the activation of RhoG and PI3K.

Materials and methods

Plasmids and antibodies

The expression plasmid encoding class Ia PI3K catalytic subunit p110α was a gift from Dr. T. Katada (University of Tokyo, Tokyo, Japan). pCAG vector encoding enhanced yellow fluorescent protein (YFP) was a gift from Drs. J. Miyazaki (Osaka University, Osaka, Japan) and T. Saito (Chiba University, Chiba, Japan). Plasmids expressing Myc-tagged EphA2, EphA2-ΔKD (amino acids 1–606, 906–976), EphA2-ΔSAM (amino acids 1–886), RhoG-V12 (G12V), Flag-tagged Ephexin4, Ephexin4-ΔDH (amino acids 1–275, 481–709), p110α fused with the CAAX sequence at the COOH terminus (p110α-CAAX), and HA-tagged Akt fused with the Src myristoylation signal at the NH2 terminus (Myr-Akt) were generated as described previously [20,41,47–49]. In anoikis experiments, we used a double promoter vector, encoding YFP and a short hairpin RNA (shRNA), or YFP and wild-type Ephexin4 (Ephexin4-WT), to express YFP protein and an shRNA or Ephexin4-WT in the same cells, as described previously [29,41,50]. The shRNAs for control luciferase, human Ephexin4, RhoG, EphA2, and ELMO2 were designed to target 19 nucleotides as described previously [22,41].

The following antibodies were used in this study: a mouse monoclonal antibody (mAb) against Myc, a rabbit polyclonal antibody (pAb) against EphA2 (C-20), and a rat mAb against RhoG (1F3 B3 E5) (Santa Cruz Biotechnology); mouse mAbs against Flag (M2) and α-tubulin (Sigma); rabbit pAbs against Akt, phosphorylated Akt (serine 473), and cleaved caspase-3 (aspartic acid 175) (Cell Signaling); a goat pAb against ELMO2 (Abcam); and secondary antibodies conjugated to horseradish peroxidase (DAKO). Anti-Ephexin4 antibody was described previously [41].

Cell culture and transfection

HeLa, MCF7, and MDCK cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 4 mM glutamine, 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin under humidified air containing 5% CO_2 at 37 °C. Cells were transfected with indicated expression vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In some experiments, HeLa cells transfected with shRNA expression vectors were used after selection with 300 μg/ml of hygromycin-B. To examine the involvement of PI3K and Akt, cells were treated with LY294002 (a PI3K specific inhibitor, Calbiochem, 20 μM) and Akt inhibitor IV (an Akt specific inhibitor, Calbiochem, 1 μM), respectively.

Anoikis assay

Cells were detached from tissue culture plates with 0.01% EDTA in phosphate-buffered saline (PBS) and cultured in complete medium in 24-well plates that had been coated with polyhydroxyethylmethacrylate (poly-HEMA) at a density of 5×10^4 cells/well. They were then collected and fixed in 4% paraformaldehyde in PBS for 15 min. After washing once with PBS, they were incubated with Hoechst 33258 (Molecular Probes) in PBS. Hoechst staining in YFP-positive single cells but not from cell aggregates was analyzed with a Leica DC350F digital camera system equipped with a Nikon Eclipse E800 microscope.