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PKA and actin play critical roles as downstream effectors in MRP4-mediated regulation of fibroblast migration

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ABSTRACT

Multidrug resistance protein 4 (MRP4), a member of the ATP binding cassette transporter family, functions as a plasma membrane exporter of cyclic nucleotides. Recently, we demonstrated that fibroblasts lacking the *Mrp4* gene migrate faster and contain higher cyclic-nucleotide levels. Here, we show that cAMP accumulation and protein kinase A (PKA) activity are higher and polarized in $Mrp4^{-/-}$ fibroblasts, versus $Mrp4^{+/+}$ cells. MRP4-containing macromolecular complexes isolated from these fibroblasts contained several proteins, including actin, which play important roles in cell migration. We found that actin interacts with MRP4, predominantly at the plasma membrane, and an intact actin cytoskeleton is required to restrict MRP4 to specific microdomains of the plasma membrane. Our data further indicated that the enhanced accumulation of cAMP in $Mrp4^{-/-}$ fibroblasts facilitates cortical actin polymerization in a PKA-dependent manner at the leading edge, which in turn increases the overall rate of cell migration to accelerate the process of wound healing. Disruption of actin polymerization or inhibition of PKA activity abolished the effect of MRP4 on cell migration. Together, our findings suggest a novel cAMP-dependent mechanism for MRP4-mediated regulation of fibroblast migration whereby PKA and actin play critical roles as downstream effectors.

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1. Introduction

Cell migration is a carefully orchestrated process requiring cross talk between cell adhesion, cytoskeleton re-organization and membrane extensions [1,2]. Conventionally, the process of cell migration consists of four distinct steps; 1) formation of leading-edge protrusions, 2) adhesion of the leading edge to the extracellular matrix, 3) forward translocations of the cell body and 4) retraction of the trailing edge by releasing attachment at the cell rear [1,2]. The spatial segregation of the intracellular signaling can dynamically regulate diverse biochemical events that ensure cell polarization and efficient cell migration [3,4]. The extracellular signal can be sensed and communicated inside the cell by specific receptors, which in turn directs the cell to extend membrane protrusions via actin polymerization [2]. Establishment of polarity is very important for directional cell migration and the spatial distribution of signaling molecules, and compartmentalized signaling plays a critical role in directing cell movement [5]. For example, adhesive biomolecules aggregate at the cell front to promote tethering of the cell protrusions to the substratum, whereas the removal of those molecules from the cell posterior facilitates detachment of the trailing edge [6]. In addition, the preferential contraction of actomyosin at the cell front pulls the cell body in a forward direction. However, several regulatory steps are involved in directional cell migration and dysregulation of any of those steps leads to catastrophic consequences [1].

Localized increases in cAMP concentration and cAMP-dependent protein kinase A (PKA) at the leading edge both play pivotal roles in ensuring the polarity of migrating cells [3,4,7]. The diversity of PKA substrates permits the regulation of multiple signaling events, based on the subcellular localization of PKA [8]. At the leading edge, PKA activates small GTPases called Ras-related C3 botulinum toxin substrate (Rac) and cell division control protein 42 homolog or Cdc42 by phosphorylation, which subsequently activates localized actin-related protein 2/3 (Arp 2/3) in a Wiskott–Aldrich syndrome proteins (WASP)/WASP-family verprolin-homologous protein or WAVE-dependent manner [9,10]. Activated Arp 2/3 binds to the sides or tips of the pre-existing actin filaments and induces the formation of new actin filaments from

Abbreviations: MEFs, mouse embryo fibroblasts; MRP4, multidrug resistance protein 4; PKA, protein kinase A; Rac, Ras-related C3 botulinum toxin substrate; SPT, single-particle tracking; VASP, vasodilator-stimulated phosphoprotein; WASP, Wiskott–Aldrich syndrome proteins.

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the pointed end at a 70° angle. The formation of branched dendritic networks of actin filament promotes a flat protrusion called lamellipodia [2,11,12]. Another PKA substrate, vasodilator-stimulated phosphoprotein (VASP) regulates actin polymerization as an anti-capping agent, and phosphorylation of VASP can regulate the oscillatory cycles of membrane extension and retraction [9,13,14].

Multidrug resistance protein 4 (MRP4) is a member of the ATP binding cassette or ABC transporter family that localizes to the plasma membrane and functions as an endogenous transporter of cyclic nucleotides. Therefore MRP4 can regulate various cyclic nucleotide dependent signaling events by modulating intracellular cAMP and cGMP levels. Recently, we demonstrated that *Mrp4*$^{-/-}$ fibroblasts migrated faster compared to *Mrp4*$^{+/+}$ fibroblasts and this was associated with moderately higher levels of total intracellular cyclic nucleotides. We also found that inhibition of MRP4 enhances the compartmentalized cAMP levels at or near the leading edge of a migrating cell [15]. Therefore, we hypothesized that this polarized elevation of cAMP is responsible for localized PKA activation at the cell front, which is the early hallmark step in directional cell migration [3,7]. In this study, we identified actin as an integral part of the MRP4 interactome. In previous studies, cAMP/PKA has been found to regulate actin polymerization and therefore the overall cell migration [16–18]. For this study, we investigated the role of MRP4 in regulating actin dynamics at the leading edge of migrating cells and the relationship between MRP4 and PKA in this process of regulation. Together, our data suggest a novel cAMP-dependent mechanism for MRP4-mediated regulation of fibroblast migration where PKA and actin play critical roles as downstream targets.

2. Materials and methods

2.1. Reagents

MRP4 inhibitor MK571 was obtained from Cayman Chemical (Ann Arbor, MI). Forskolin was obtained from Tocris (Ellisville, MO). IBMX, Lat B, cpt-cAMP, and cpt-cGMP were purchased from Sigma-Aldrich (St. Louis, MO). Zaprinast and PKA inhibitor H-89, 2HCl were purchased from Enzo Life Sciences (Farmingdale, NY).

2.2. Cell culture and transfections

MEF cells and NIH 3T3 cells were cultured in DMEM media (MRP4 overexpressed) and HEK293 cells were grown in DMEM F-12 media (Invitrogen; Carlsbad, CA), both containing 10% FBS and 1% penicillin/

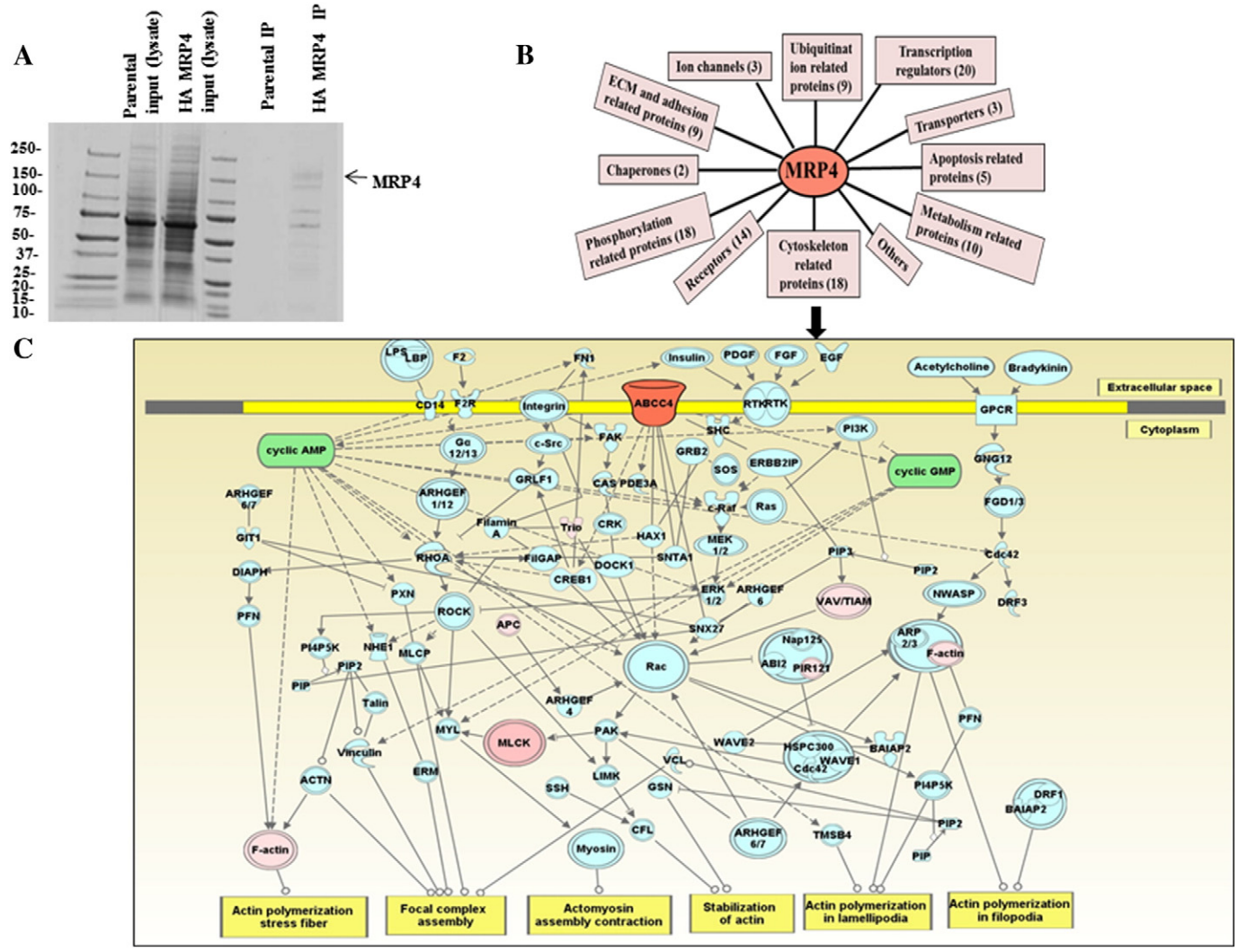

Fig. 1. Actin is an integral part of the MRP4 interactome. (A) MRP4 was affinity purified using anti-HA beads from HA-MRP4-overexpressing HEK293 cells. The Coomassie-stained gel shows the immunoprecipitated full-length HA-MRP4 and its interacting partners. (B) MRP4-interacting partners were identified by mass-spectrometric analysis and categorized based on their molecular function. (C) Ingenuity pathway analysis showed the interconnectivity between the MRP4 interactome components in the context of cell migration. We assigned MRP4 as seed (orange) and the MRP4-interacting proteins as high-confidence interactome (pink) and indicated the intermediate proteins involved in the pathways based on the literature and experimental evidence (blue).

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