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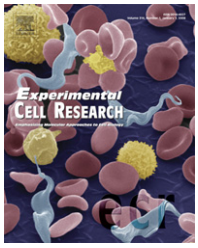

Research Article

Clathrin dependent endocytosis of E-cadherin is regulated by the Arf6GAP isoform SMAP1

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ABSTRACT

E-cadherin is a central component of the adherens junction in epithelial cells and continuously undergoes endocytosis via clathrin-coated vesicles and/or caveolae depending on the cell type. In this study, we examined the role of SMAP1, a clathrin-interacting GTPase-activating protein (GAP) for the ADP-ribosylation factor 6 (Arf6) GTPase, in E-cadherin endocytosis. Mardin–Darby canine kidney (MDCK) epithelial cells were used as a model, and SMAP1 localized in the cytoplasm and along the adherens junction where E-cadherin was present. Next, activity of SMAP1 was compared with that of other Arf6GAPs (and/or an effector of Arf6-GTP), namely GIT1 and AMAP2/DDEF2. Overexpression of SMAP1 but not GIT1 nor AMAP2/DDEF2 strongly inhibited basal, as well as phorbolester-induced, internalization of E-cadherin. Notably, AMAP2/DDEF2 rather enhanced the caveolae-mediated incorporation of a membrane protein other than E-cadherin. Thus, in MDCK cells, E-cadherin appeared to be endocytosed solely through SMAP1-regulated clathrin-coated vesicles. Furthermore, MDCK cells overexpressing SMAP1 showed a reduced degree of cell migration compared to untransfected cells, as assessed by wound healing and Transwell assays, and this reduction in migration appeared to be due to the accumulation of E-cadherin at the adherens junction in cells overexpressing SMAP1. Collectively, SMAP1 likely represents a key Arf6GAP in clathrin dependent endocytosis of E-cadherin in MDCK cells. This activity of SMAP1 in E-cadherin turnover may be involved in epithelial organization and/or epithelial–mesenchymal transition.

Introduction

E-cadherin is a central component of the adherens junction in epithelial cells and is essential for the organization of epithelial tissues. The extracellular domains of E-cadherin on neighboring cells interact with each other in a Ca^{2+}-dependent, homophilic manner. The structure of the adherens junction is disrupted in the epithelial–mesenchymal transition (EMT) that is involved in phenomena such as gastrulation, tissue reorganization, and malignant transformation [1]. Down-regulation of E-cadherin results in dissolution of the adherens junction, which reduces cell–cell interactions and eventually results in cell motility [2]. The amount of E-cadherin residing in the adherens junction is controlled by transcriptional, as well as

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post-transcriptional, mechanisms [3,4]. Transcriptional mechanisms include expression of several transcription factors such as Snail, SIP1 and Twist that are up-regulated during the EMT [5]. These transcription factors bind to the E-box element in the promoter of the *E-cadherin* gene and suppress its transcription [6]. Post-transcriptionally, endocytosis of E-cadherin is enhanced accompanying the induction of EMT, and contributes to changes during the early phases of EMT [7]. The endocytosed E-cadherin is mono-ubiquitinated by the E3 ubiquitin ligase Hakai, targeted to lysozomes, and degraded there [8,9]. Thus, elucidating the molecular mechanisms involved in E-cadherin endocytosis is a key element in understanding the process of EMT.

ADP-ribosylation factors (Arfs) are Ras-related small GTPases found in all eukaryotic organisms [10–13]. In mammals, 6 Arfs (Arf1 to 6) and several Arf-related proteins have been identified. Arf1 functions in vesicle transport in the Golgi-to-endoplasmic reticulum, inter-Golgi and *trans*-Golgi-network-endosome pathways. Arf6 functions in endocytosis of membrane proteins and recycling back to the plasma membrane. Like other small GTPases, Arfs cycle between a GTP-bound active form and a GDP-bound inactive form. A specific guanine nucleotide-exchange factor (GEF) converts Arfs to an active form, whereas a specific GTPase-activating protein (GAP) activates the GTPase of Arfs and converts them to an inactive form.

Recently, we identified SMAP1, a novel Arf6 specific GAP [14]. Overexpression of SMAP1 in HeLa cells abrogates the clathrin dependent internalization of the transferrin receptor. SMAP1 possesses a consensus clathrin-binding motif and is indeed capable of binding to the clathrin heavy chain directly. Thus, we have proposed that SMAP1 regulates clathrin dependent endocytosis as an Arf6GAP. There are several other members in the Arf6GAP family, including AMAP2/DDEF2/PAG3/Papα/ASAP2, GIT1/2, ACAP1/2 and ARAP2 (note that AMAP2/DDEF2 is reported to function rather as an effector of Arf6-GTP; see Refs. [15,16]). Among these Arf6GAPs, AMAP2/DDEF2 is involved in clathrin independent endocytosis of Fcγ receptor and CD25 [15,17] and the GITs regulate endocytosis of the epidermal growth factor receptor and several G-protein coupled receptors in a clathrin- and ligand binding-dependent fashion [18–20]. These studies indicate that there are multiple GAPs specific for Arf6 and that distinct isoforms of Arf6GAPs are used in different endocytic pathways, depending on the type of cargo protein [21,22].

Mardin–Darby canine kidney (MDCK) cells have been used extensively to study E-cadherin endocytosis because of their epithelial nature. There are several lines of evidence indicating that endocytosis of E-cadherin follows a pathway similar to transferrin endocytosis. First, hypotonic treatment or overexpression of the ENTH domain of epsin, a clathrin-adaptor, both block clathrin dependent endocytosis and, concomitantly, abrogate the endocytosis of E-cadherin in MDCK cells [23,24]. Secondly, transfection of cells with Arf6T27N, a dominant negative mutant of Arf6, also inhibits E-cadherin endocytosis [25,26]. Thirdly, under unstimulated, constitutive conditions, internalized E-cadherin is recycled back to the plasma membrane via an early endosome-to-recycling endosome pathway, similar to the transferrin receptor [23,27]. In fact, a fraction of internalized E-cadherin colocalizes with internalized transferrin receptors [25]. Thus, endocytosis of E-cadherin appears to occur through an Arf6 and clathrin dependent pathway at least in the case of MDCK cells. It must be noted that another route of E-cadherin endocytosis, namely via caveolae, is also reported for the other epithelial cell lines [28,29].

We hypothesized that SMAP1 regulates the endocytosis of E-cadherin in MDCK cells. In this study, we compared the activity of SMAP1 with that of other Arf6GAPs. In MDCK cells, SMAP1 is involved in clathrin dependent endocytosis of E-cadherin, whereas AMAP2/DDEF2 is in the caveolin-dependent endocytosis of another membrane protein. Furthermore, our data suggest that SMAP1 may be involved in the EMT.

Materials and methods

Reagents and antibodies

12-O-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma (St. Louis, MO). The antibodies used were the anti-influenza hemagglutinin (HA) rat monoclonal antibody (mAb) 3F10 from Roche Diagnostics (Indianapolis, IN), the mouse mAbs against β-actin (AC-15) and vimentin (V9) from Sigma, the mouse mAb against clathrin heavy chain (X22) from Affinity BioReagents (Golden, CO), the mouse mAb against E-cadherin from BD Transduction Laboratories (Lexington, KY), the rabbit polyclonal antibody against caveolin-1 (N-20) from Santa Cruz Biotechnology (Santa Cruz, CA), the Cy3-conjugated goat anti-rat and donkey anti-rabbit IgGs from Chemicon (Temecula, CA), and the Alexa488-conjugated goat anti-mouse, anti-rabbit and anti-rat IgGs from Molecular Probes (Eugene, OR).

Plasmid construction

The pcDNA3 expression vectors (Invitrogen, Carlsbad, CA) harboring HA-tagged *SMAP1* and *SMAP1R61Q* have been described previously [14]. The cDNAs for *GIT1* and *Arf6* were amplified from first-strand cDNAs prepared from murine brain and the mutations were introduced by PCR. *GIT1* and *Arf6* were HA-tagged at their amino-termini and inserted into pcDNA3 expression vectors. pEGFP-C1/AMAP2/DDEF2 and pEGFP-C1/AMAP2/DDEF2C436A have been described previously [17]. pcDNA3/DynaminII K44A was kindly provided by K. Nakayama (Kyoto University, Kyoto, Japan).

MDCK cells and its derivative clones overexpressing SMAP1

MDCK cells were obtained from the RIKEN cell bank (Tsukuba, Japan) and cultured in DMEM supplemented with 10% (v/v) FBS and 50 μg/ml gentamycin. The cells were grown on plastic dishes unless specifically indicated. To obtain MDCK cell clones overexpressing SMAP1, cells were transfected with a plasmid harboring *HA-SMAP1* and a neomycin-resistance gene and cultured in DMEM supplemented with 1mg/ml G418. Drug-resistant clones were isolated using a penicillin cup, expanded, and stocked.

Immunofluorescence microscopy

Subconfluent MDCK cells on coverslips were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) according