EXPERIMENTAL CELL RESEARCH 317 (2011) 539–551

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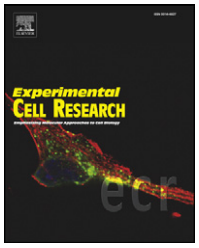

Research Article

Numb is a negative regulator of HGF dependent cell scattering and Rac1 activation

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ARTICLE INFORMATION

Article Chronology:
Received 11 October 2010
Revised version received
4 December 2010
Accepted 6 December 2010
Available online 11 December 2010

Keywords:
Numb
E-cadherin
Cell adhesion
Hepatocyte growth factor
Rac1
EMT

ABSTRACT

Numb is an endocytic adaptor protein that regulates internalization and post-endocytic trafficking of cell surface proteins. In polarized epithelial cells Numb is localized to the basolateral membrane, and recent work has implicated Numb in regulation of cell adhesion and migration, suggesting a role for Numb in epithelial–mesenchymal transition (EMT). We depleted MDCK cells of Numb and examined the effects downstream of EMT-promoting stimuli. While knockdown of Numb did not affect apicobasal polarity, we show that depletion of Numb destabilizes E-cadherin-based cell–cell adhesion and promotes loss of epithelial cell morphology. In addition, Numb knockdown in MDCK cells potentiates HGF-induced lamellipodia formation and cell dispersal. Examination of Rac1-GTP levels in Numb knockdown cells revealed hyperactivation of Rac1 following extracellular calcium depletion and HGF stimulation, which corresponds with enhanced loss of cell adhesions and lamellipodia formation. Furthermore, inhibition of Rac1 in Numb depleted cells stabilized cell–cell contacts following depletion of extracellular calcium. Together, these data indicate that Numb acts to suppress Rac1-GTP accumulation, and its loss leads to increased sensitivity toward extracellular signals that disrupt cell–cell adhesion to induce epithelial–mesenchymal transition (EMT) and cell dispersal.

Introduction

Epithelial–mesenchymal transition (EMT) involves highly regulated events that convert epithelial cells to mesenchymal cells. During EMT, non-motile, polarized epithelial cells dissolve cell–cell junctions, lose apical–basal polarity, and adopt a motile mesenchymal phenotype. While EMT is required during embryonic development, deregulated EMT in adult organisms can also participate in tumourigenesis [1,2]. Given the importance of cell–cell interactions in the formation of polarized epithelial cells, many studies have focused on understanding the regulation of adhesion molecules with respect to EMT. E-cadherin, in particular, has received much attention since its identification as a suppressor of tumour cell invasion [3]. Indeed, loss of E-cadherin expression occurs in many cancers including, prostate and breast cancers [4,5]. Endocytosis of E-cadherin is one way of attenuating E-cadherin adhesive function [6]. Following hepatocyte growth factor (HGF) stimulation, E-cadherin is co-endocytosed with the receptor tyrosine kinase, Met [7]. HGF stimulation promotes ubiquitination of E-cadherin by the E3 ubiquitin ligase, Hakai, resulting in E-cadherin internalization [8], indicating that growth factor signalling and surface expression of adhesion molecules are tightly coupled events.

Numb is an evolutionarily conserved membrane-associated endocytic adaptor protein that controls cell fate. Numb is down-regulated in human lung cancer, breast cancer, and medulloblastoma [9–11]. A tumour suppressor function for Numb has also been defined, which is attributed, in part, to its role in stabilizing p53 [12].

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0014-4827/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2010.12.005

However, given its involvement in multiple signalling pathways, including those functioning downstream of Notch and Hedgehog, [10,13], it is likely that Numb participates in additional pathways to exert its tumour suppressive function.

In polarized epithelial cells, Numb localization is restricted to the basolateral membrane [14], and Numb has been reported to regulate localization of E-cadherin [15,16]. Indeed, Numb and the related protein, Numblike, are required to maintain cadherin-based adhesions in neuroepithelial cells. Furthermore, Numb also interacts with and influences endocytosis of β1-integrin, thereby regulating cell migration [17,18]. These studies raise the possibility that Numb functions to regulate cell–cell adhesion as well as adhesion to the extracellular matrix (ECM). As the disruption of cell adhesion is an early step in epithelial–mesenchymal transition (EMT), Numb may function as a negative regulator of tumour progression by inhibiting EMT. Here we provide evidence that Numb functions to inhibit early steps in EMT by stabilizing cell–cell adhesion, and suppressing cell spreading and dispersal. Furthermore, we report that Numb functions to suppress Rac1 activation in this context. Our studies provide insight into the role of Numb in tumour suppression, and present evidence that Numb functions to stabilize cell adhesion by regulating Rac1 activity.

Materials and methods

Cell culture, transfection, and generation of MDCK-NbshRNA stable lines

MDCK II cells were maintained in DMEM (Wisent) supplemented with 5% FBS. Generation of stable lines was performed as follows: MDCK cells were transfected with 2 μg of a short hairpin construct targeting Numb: 5′-TTAAAGGGTGACATCTTCTGGCT-3′ (pSM2C, Open Biosystems, V2HS_23979) or control luciferase with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Following selection in 2 μg/mL puromycin for 2 weeks, colonies were isolated and propagated to generate stable cell lines.

For transient knockdown of Numb, MDCK cells were transfected with 200 pmol of siRNA targeting Numb: 5′-GCACCTGCCCAGTGGATCC-3′ or a distinct sequence previously described in [19], or scrambled RNA control (Dharmacon) using Lipofectamine 2000 according to the manufacturer's protocol. Experiments were conducted 48 h post-transfection.

For stable rescue cell lines, we generated silent mutations in the shRNA target regions of Numb-p72 (Flag-Nbp72-silent) using QuikChange XL site-directed mutagenesis kit (Stratagene) and the following primers: 5′-GGA TTT CCT GCT CTT TCT CAG AAA ATG AGC CCC TTT AAA CGC CAG-3′ and 5′-CTG GCG TTT AAA GGG GCT CAT TTT CTG AGA AAG AGC AGG AAA TCC-3′. To generate stable rescue cell lines, MDCK-NbshRNA cells were transfected with Flag-Nbp72-silent, or empty Flag vector, and pGK-Hygro construct in a ratio of 8:1 with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Following selection in 500 μg/mL hygromycin for 2 weeks, colonies were isolated and propagated to generate stable cell lines.

3-dimensional MDCK cysts were cultured as described in [20] with some modifications. Briefly, MDCK cells were trypsinized and 2500 cells were seeded to an 8-well glass chamberslide (LabTek) coated with Growth Factor-Reduced Matrigel (BD Biosciences). Cells were cultured in 2% Matrigel in normal growth media for 10 days, and media were replaced every 2–3 days.

Calcium switch experiments

Cells were seeded at a density of 10^6 on Falcon Cell Culture Inserts and grown for 2 days. Cells were serum starved for 1–2 h followed by addition of DMEM containing 4 mM EGTA and 0.1% dialyzed FBS for various timepoints. For Rac1 inhibition studies, cells were pre-incubated in DMEM containing 100 μM NSC23766 (Calbiochem) for 30 min followed by addition of DMEM containing 4 mM EGTA, 0.1% dialyzed FBS, and 100 μM NSC23766. Cells were fixed in 2% PFA containing 30 mM sucrose for 20 min, permeabilized in 0.05% saponin, and blocked in 3% normal donkey serum. Primary antibodies were applied for 1 h at 37 °C. Cells were washed as follows: 1×10 min with PBS, 3×10 min with 0.05% Triton in PBS, and 1×10 min with PBS. Secondary antibodies were applied for 30 min at 37 °C; cells were washed as described above, and mounted in Dako Cytomation fluorescent mounting medium. Confocal images were acquired using a Zeiss LSM510 confocal microscope at 100× magnification or a Quorum spinning disk confocal microscope at 63× magnification and image analysis was performed using Volocity software.

E-cadherin trafficking experiments

E-cadherin surface labelling was performed as described in McGill et al. [21] with some modifications. Briefly, MDCK cells were cooled to 4 °C for 1 h, washed with cold PBS, and labelled with EZ-link NSSH-biotin (Pierce) in biotinylation buffer (154 mM NaCl, 10 mM HEPES, 3 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM glucose, pH 7.6) for 1 h at 4 °C. Cells were then washed twice in cold PBS and incubated in 100 mM Glycine in DMEM + 5% FBS on ice for 10 min to quench unconjugated biotin. At this point, one plate from each set was lysed in RIPA buffer to measure total surface biotinylation. A second plate from each set was used to measure efficiency of MeSNa (2-mercaptoethanesulfonic acid) stripping of biotinyl groups from the cell surface by four 15-minute incubations in 50 mM MeSNa in stripping buffer (100 mM NaCl, 50 mM Tris–HCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 8.6) at 4 °C prior to lysis. The remaining plates were incubated at 18 °C or 37 °C in DMEM containing 4 mM EGTA and 0.1% dialyzed FBS for various timepoints. Each plate was stripped of surface biotin as described above and lysed in RIPA buffer. Equal amounts of total protein lysate were incubated with streptavidin-sepharose beads (Pierce) overnight at 4 °C to isolate biotinylated proteins and washed 3 times in NP40 wash buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM EGTA, 10% glycerol, 1.5 mM $CaCl_2$, 1% NP40). Isolated biotinylated proteins were analyzed with SDS-PAGE and biotinylated E-cadherin was visualized by immunoblotting with anti-E-cadherin antibody.

Rac activation assay

Pak1 p21-binding domain (PBD) was subcloned from a GFP-PBD construct (gift from S. Grinstein) into pGEX4T3 and expressed in *E. coli* (BL21). GST-PBD was purified in PLC lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 10 mM sodium pyrophosphate, 5 mM sodium orthovanadate, protease inhibitors (Roche)) containing 2 mM DTT and bound to Glutathione-Sepharose 4B beads (Amersham) in Binding Buffer (25 mM Tris pH 7.4, 30 mM $MgCl_2$, 40 mM