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Research paper

WIP/ITSN1 complex is involved in cellular vesicle trafficking and formation of filopodia-like protrusions

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ABSTRACT

WIP (WASP interacting protein) together with N-WASP (neural Wiskott-Aldrich syndrome protein) regulates actin polymerization that is crucial for invadopodia and filopodia formation. Recently, we reported the WIP interaction with ITSN1 which is highly implicated in endo-/exocytosis, apoptosis, mitogenic signaling and cytoskeleton rearrangements. Here we demonstrate that the WIP/ITSN1 complex is involved in the transferrin receptor recycling and partially co-localizes with a marker of the fast recycling endosomes, RAB4. Moreover, ITSN1 recruits WIP to RAB4-positive vesicles upon overexpression. Our data indicate that WIP enhances the interaction of N-WASP with ITSN1 and promotes ITSN1/β-actin association. Moreover, the WIP/ITSN1-L complex facilitates formation of filopodia-like protrusions in MCF-7 cells. Thus, WIP/ITSN1 complex is involved in the cellular vesicle trafficking and actin-dependent membrane processes.

1. Introduction

Actin cytoskeleton remodeling is indispensable for most cellular processes such as cell polarity, proliferation, migration, endo- and exocytosis. WIP (WASP interacting protein) plays an important role in actin-mediated cellular processes. This is a ubiquitously expressed 503 aas protein of the verprolin family which consists of the N-terminal VH (verprolin homology) domain, containing two actin-binding WH2 (WASP Homology 2) domains, followed by a range of proline-rich motifs that mediate interaction with a majority of WIP partners, and the C-terminal WASP binding domain (WBD). WIP also contains three profilin-binding ABM-2 (actin-based motility 2) domains (Fried et al., 2014). Through the WBD domain, WIP interacts with its constitutive partner Neural Wiskott-Aldrich syndrome protein (N-WASP), an actin nucleation-promoting factor (Martinez-Quiles et al., 2001; Moreau et al., 2000). WIP regulates stability, location and function of N-WASP (Anton et al., 2007). Both WIP and N-WASP play crucial roles in the cellular actin Arp2/3-dependent dynamics that are essential for cell motility and cancer cell invasion (Yamaguchi et al., 2005; Garcia et al., 2014). WIP is highly implicated in the extracellular matrix degradation promoting invadopodia formation (actin-rich cancer cell protrusions with proteolytic activity) (Garcia et al., 2016). Moreover, WIP is involved in malignant tumorigenesis: low WIP expression levels are associated with favorable prognosis for patients with breast, colon and brain cancers (Staub et al., 2009).

Recently, we demonstrated that WIP interacts with the scaffold protein ITSN1 (Gryaznova et al., 2015). ITSN1 is a multi-domain protein involved in endo- and exocytosis, cellular signaling, cell survival and actin cytoskeleton remodeling. In addition, ITSN1 is associated with the progression of several neurodegenerative pathologies and is implicated in cancer cell survival and migration (O'Bryan, 2010; Tsyba et al., 2011). ITSN1 is an essential component of the endocytic machinery in neuronal and non-neuronal cells (Hussain et al., 1999). It has two main isoforms, short and long, generated by alternative splicing. The short isoform (ITSN1-S) contains two EH (Eps15 homology) domains, a coiled-coil (CC) domain and five SH3 (src homology 3) domains (O'Bryan et al., 2001). The long isoform (ITSN1-L) is co-linear with ITSN-S, but has an extended C-terminus encoding the DH (Dbl homology) domain, the PH (pleckstrin homology) domain, and the C2 domain. ITSN1-L expression is brain specific where it participates in dendritic spine development and synaptic vesicle trafficking (Irie and Yamaguchi, 2002; Nishimura et al., 2006; Pechstein et al., 2010). However, ITSN1-L is also expressed at low levels in cancer cell lines (Novokhatska et al., 2013; Kropyvko et al., 2016).

We have previously demonstrated that ITSN1 and WIP co-localize at the invadopodia formation sites and in the clathrin-coated pits

Abbreviations: ITSN1, intersectin 1; MMPs, matrix metalloproteases; N-WASP, neural Wiskott-Aldrich syndrome protein; SCC, Spearman correlation coefficient; WIP, WASP-interacting protein

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(Gryaznova et al., 2015). In the present study, we further investigate the cellular functions of the WIP/ITSN1 complex and demonstrate that the WIP/ITSN1-L complex overexpression rescues transferrin trafficking. Furthermore, we conclude that WIP/ITSN1-L complex is involved in RAB4-dependent vesicle recycling and induces filopodia-like protrusions formation.

2. Materials and methods

2.1. Plasmid constructs

The DNA expression constructs encoding HA-WIP, HA-WIPΔWBD, Omni-N-WASP, GST-fused five SH3 domains of ITSN1 (ITSN1 SH3(A-E)), GFP-ITSN1-L and Omni-ITSN1-L were described previously (Gryaznova et al., 2015; Tsyba et al., 2008; Morderer et al., 2012; Gubar et al., 2012). GFP-RAB4 was kindly provided by Dr. Serhiy Havrylov (Warsaw, Poland). pCMV-HA (Clontech), pcDNA4/HisMax (Omni) (Invitrogen), pGEX-4T-3(GST) (GE Healthcare) and pEGFP-C1 (GFP) (Clontech) vectors were used as controls.

2.2. Cell culture

MCF-7 and 293 cell lines were obtained from Bank of cell lines of the R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NASU (Ukraine). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Sigma-Aldrich), 50 U/ml penicillin and 100 μg/ml streptomycin. The cells were transiently transfected using JetPEI® (polyethyleneimine, Polyplus Transfection) according to manufacturer's recommendations and further processed 24 h after transfection.

2.3. Antibodies

Rabbit polyclonal antibodies against the EH2 domain of human ITSN1 (anti-ITSN1) were described previously (Nikolaienko et al., 2009). Polyclonal goat anti-WIP (G-20) and polyclonal rabbit anti-Omni (M-21) antibodies were purchased from Santa Cruz Biotechnology. Monoclonal anti-HA and anti-β-actin antibodies were from Covance and Sigma-Aldrich, respectively. Secondary horseradish peroxidase-labeled anti-rabbit and anti-mouse antibodies were from Promega; donkey anti-goat alexa Fluor 633, donkey anti-mouse Alexa Fluor 647, goat anti-rabbit Alexa Fluor 555, goat anti-rabbit Alexa Fluor 488, donkey anti-mouse Alexa Fluor 555 and Alexa 555-phalloidin were from Invitrogen.

2.4. Protein expression, GST pull down and Western blot

Protein expression, GST pull downs and Western blots were performed as described previously (Gryaznova et al., 2015). Briefly, GST-fusion proteins were expressed in *Escherichia coli* BL21(DE3) strain and purified with glutathione-Sepharose beads 4B (GE Healthcare) according to the manufacturer's instructions. For pull-down experiments, 5–10 μg of GST or GST-fused proteins bound to glutathione-Sepharose 4B were incubated for 1 h at +4 °C with the precleared cell lysates (2 mg of proteins). The beads were washed three times and eluted with Laemmli sample buffer. The proteins were resolved in SDS–PAGE, transferred to nitrocellulose membranes (Bio-Rad) and blocked in 5% nonfat milk. The membranes were incubated with primary antibodies for 1 h followed by incubation with peroxidase-conjugated secondary antibodies for 40 min. Immunoreactive bands were detected using ECL™ reagents (Sigma-Aldrich). Chemiluminescence was captured with Molecular Imager ChemiDoc™ XRS+ (Bio-Rad). The density of the Western blot bands was calculated in ImageJ (developed at the U.S. National Institutes of Health (http://rsb.info.nih.gov/ij/)) and correlated with the amount of GST-SH3 domains. Data visualization and statistical analysis were performed in MS Excel or OriginPro 9.1.

2.5. Immunostaining and confocal microscopy

The cells were plated on coverslips and transfected with the appropriate plasmid DNA using the JetPEI® reagent (Polyplus Transfection). 24 h post-transfection, the cells were washed with ice-cold PBS (phosphate buffered saline), fixed in 4% formaldehyde for 15 min and washed three times in PBS containing 0.2% Triton X-100. The cells were blocked with 2% bovine serum albumin (BSA), 0.2% Triton X-100 in PBS for 30 min at room temperature and incubated with the appropriate antibodies. Cell nuclei were stained with Hoechst 33258 (Sigma-Aldrich). The slides were mounted in Mowiol (Sigma-Aldrich) and confocal images were taken using Zeiss LSM510 confocal microscope.

2.6. Transferrin internalization assay

24 h after transfection the 293 cells grown on coverslips were rinsed twice with PBS and serum-starved for 2 h. The cells were then incubated with 50 mg/ml Alexa 633-conjugated transferrin (Sigma-Aldrich) in serum-free DMEM for 30 min at 37 °C. Subsequently, coverslips were rinsed twice with PBS, fixed in 4% formaldehyde and processed for immunocytochemistry as described above. Perinuclear transferrin localization was estimated as a histogram profile on the cell equator using ImageJ software.

2.7. Analysis of co-localization

Protein co-localization analysis was performed as described previously (Kropyvko et al., 2017). Briefly, the SCC (Spearman correlation coefficient) was calculated using PSC (Pearson-Spearman correlation) co-localization plugin in ImageJ 1.37c software according to the procedure of French et al., 2008 (French et al., 2008).

2.8. Filopodia-like protrusions calculation

The filopodia-like protrusions were visualized with Alexa Fluor 555-conjugated phalloidin (Invitrogen). The number of filopodia per cell was calculated using FiloQuant plugin in ImageJ (Jacquemet et al., 2017). Data visualization and statistical analysis were performed in MS Excel or OriginPro 9.1.

2.9. Data analysis

All data presented were quantified as mean ± SD. Statistical tests used were One-Way ANOVA with *post-hoc* Fisher test, Mann-Whitney *U* test or paired sample *t*-test in Origin software 9.1.0 (OriginLab). Differences between groups were considered statistically significant if $p < 0.05$.

3. Results

3.1. Overexpression of WIP/ITSN1 complex rescues intracellular transferrin distribution in 293 cells

ITSN1 plays an important role in endocytosis and vesicle trafficking in mammals. It was demonstrated that either overexpression or silencing of ITSN1 leads to a decrease in clathrin-dependent internalization in different cell types (Sengar et al., 1999; Pucharcos et al., 2000; Martin et al., 2006; Thomas et al., 2009). The ITSN1-binding protein WIP is also involved in endocytosis. Silencing of WIP prevents endocytosis-mediated transferrin uptake in MDA-MB-231 cells (Gargini et al., 2016). Recently we demonstrated that WIP and ITSN1 proteins co-localize in clathrin-coated pits (CCPs). To determine whether the WIP/ITSN1 complex plays a role in endocytosis, we performed fluorescent transferrin uptake assays in 293 cells co-expressing HA-WIP together with Omni-ITSN1-L, each of the proteins separately or empty