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Vimentin phosphorylation and assembly are regulated by the small GTPase Rab7a

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ABSTRACT

Intermediate filaments are cytoskeletal elements important for cell architecture. Recently it has been discovered that intermediate filaments are highly dynamic and that they are fundamental for organelle positioning, transport and function thus being an important regulatory component of membrane traffic. We have identified, using the yeast two-hybrid system, vimentin, a class III intermediate filament protein, as a Rab7a interacting protein. Rab7a is a member of the Rab family of small GTPases and it controls vesicular membrane traffic to late endosomes and lysosomes. In addition, Rab7a is important for maturation of phagosomes and autophagic vacuoles. We confirmed the interaction in HeLa cells by co-immunoprecipitation and pull-down experiments, and established that the interaction is direct using bacterially expressed recombinant proteins. Immunofluorescence analysis on HeLa cells indicate that Rab7a-positive vesicles sometimes overlap with vimentin filaments. Overexpression of Rab7a causes an increase in vimentin phosphorylation at different sites and causes redistribution of vimentin in the soluble fraction. Consistently, Rab7a silencing causes an increase of vimentin present in the insoluble fraction (assembled). Also, expression of Charcot–Marie–Tooth 2B-causing Rab7a mutant proteins induces vimentin phosphorylation and increases the amount of vimentin in the soluble fraction. Thus, modulation of expression levels of Rab7a wt or expression of Rab7a mutant proteins changes the assembly of vimentin and its phosphorylation state indicating that Rab7a is important for the regulation of vimentin function.

1. Introduction

Intermediate filaments (IFs) are a major element of the cytoskeleton of animal cells although the less understood [1,2]. The IF protein family comprises about 70 different, although structurally related, members that are grouped in five different classes [1,2]. IF proteins are expressed at different stages during development and differentiation and several different IF proteins are expressed at the same time in a cell type [1,2]. IFs are the primary determinants of cell architecture and are assembled and organized in a highly dynamic way [2,3]. Recently, it has been discovered that IFs are also important for organelle positioning, organelle transport and organelle function [4].

Vimentin, the major intermediate filament protein of mesenchymal cells, is highly conserved in vertebrates, with a variable expression pattern during development and differentiation [5]. It has been demonstrated that vimentin moves bi-directionally along microtubules using kinesin and dynein–dynactin [3]. Vimentin assembly and functions are regulated by phosphorylation and indeed vimentin shows a complex phosphorylation pattern with several kinases being involved [5,6]. Different phosphorylation patterns of vimentin are associated to different mitotic phases [5,6].

A number of studies have established that vimentin has many distinct and complex functions and it seems to organize several cellular processes as attachment, migration and signaling by controlling the function of key proteins [5]. For instance, vimentin has a key role in the migration of immune cells, in the regulation of membrane-associated protein complexes, in the modulation of protein kinases serving as a scaffold, and even in the regulation of DNA by, for instance, sequestering transcription factors [5]. Also, beside regulating integrin trafficking, vimentin regulates integrin functions by constituting the vimentin associated matrix adhesions that, in some cases, involve even a direct interaction between vimentin and integrins [5,7,8]. Furthermore, vimentin plays an important role also in membrane traffic. Indeed, vimentin filaments are required for late endocytic traffic, for correct positioning of endosomes and lysosomes and for maturation of autophagosomes [9,10].

Using the two-hybrid system we isolated vimentin as an interacting partner of Rab7a, a small GTPase controlling late endosomal traffic. Unlike Rab7b, which is involved in the regulation of endosomes to TGN transport [11,12], Rab7a is important for the biogenesis of lysosomes, phagolysosomes and autolysosomes [13–16]. Rab7a exerts

Abbreviations: GST, Glutathione-S-Transferase; HA, Hemagglutinin; EGFP, Enhanced Green Fluorescent Protein

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its functions through several effector proteins, including RILP (the Rab-interacting lysosomal protein), which is important also for multivesicular body biogenesis [17–20]. Mutations in the *rab7a* gene cause a peripheral neuropathy, the Charcot–Marie–Tooth type 2b (CMT2B), characterized by prominent sensory loss, muscle weakness, muscle atrophy, recurrent infections and ulcers leading to amputations [21–25]. In the nervous system Rab7a controls endosomal trafficking and signaling of nerve growth factor receptors [26,27], and mutant Rab7a proteins causing CMT2B have altered nucleotides K_{off}, inhibit neurite outgrowth and alter neurotrophin trafficking and signaling [28–33].

Our data indicate that Rab7a interacts directly with vimentin, and that this interaction modulates vimentin phosphorylation and assembly. Thus, we have discovered a new mechanism for vimentin regulation. These data suggest that Rab7a could, through the interaction with vimentin, be involved in the regulation of new cellular processes.

2. Materials and methods

2.1. Cells and reagents

Restriction and modification enzymes were from New England Biolabs (Ipswich, MA). Chemicals and tissue culture reagents were from Sigma-Aldrich (St. Louis, MO). HeLa cells were grown in DMEM supplemented with 10% heat inactivated FBS, 2 mM glutamine, 100 U/ml penicillin and 10 mg/ml streptomycin, in a 5% CO_2 incubator at 37 °C.

2.2. Yeast two-hybrid assay

A Gal4-binding domain/Rab7a ΔC fusion construct in the pGBKT7 vector was used to screen a pretransformed normalized human Matchmaker cDNA library (Clontech, # 638874) in order to identify Rab7a interacting proteins using AH109 yeast cells [34–36]. Transformants were plated onto synthetic medium lacking His, Leu, and Trp and clones were picked up after 5–6 days of incubation at 30 °C. From 2×10^6 primary transformants, 18 were encoding true positive clones and two of them encoded vimentin. Specificity tests were made transforming AH109 yeast cells with the pGBKT7 vector or PGBKT7-RILP (as a negative controls) or the pGBKT7-Rab7a wt constructs, and with the PGADT7-vimentin constructs. Clones were then assayed for growth on selective medium and for β-galactosidase activity using *o*-nitrophenyl-β-D-galactoside as a substrate [34–36].

2.3. Plasmid construction

Rab7a constructs used in this study have been described previously [28,31,37]. Myc-tagged vimentin was obtained from OriGene (RC201546). Vimentin ORF was transferred using AsiSI and MluI restriction enzymes to pCMV6-AN-HA (OriGene, PS100013) to obtain a vector for the expression of HA-tagged vimentin. Vimentin ORF was also amplified by PCR using the following oligonucleotides: 5′-ACGCGGATCCATGTCCACCAGGTCCGTG-3′ and 5′-CCGGAATTCTTATTCAAGGTCATCGTGATGC-3′ and cloned in the vector pGEX4T3 using the restriction enzymes BamHI and EcoRI in order to obtain a plasmid for the bacterial expression of GST-tagged vimentin. All the newly made constructs were sequenced verified.

2.4. Transfection and RNA interference

Transfection was performed using Metafectene Pro or Metafectene Easy from Biontex (Karlsruhe, DE), as indicated by the manufacturer. After 20 h of transfection cells were processed for immunofluorescence or biochemical assays. For RNA interference control RNA (sense sequence 5′-ACUUCGAGCGUGCAUGGCUTT-3′ and antisense sequence 5′-AGCCAUGCACGCUCGAAGUTT-3′) and Rab7a-1 siRNAs (sense sequence 5′-GGAUGACCUCUAGGAAGAATT-3′ and antisense sequence 5′-UUCUUCCUAGAGGUCAUCCTT-3′) were purchased from MWG-Biotech (Ebersberg, Germany). The Rab7a siRNAs were efficient in silencing Rab7a as mRNA levels were reduced of about 80% and the Rab7a endogenous protein was not anymore detectable by Western blot [28]. Briefly, HeLa cells were plated 1 day before transfection in 6 cm diameter tissue culture dishes, transfected with siRNAs using Oligofectamine from Invitrogen (Milan, Italy) for 72 h, re-plated and left 48 h before performing further experiments.

2.5. Coimmunoprecipitation, pull-down and direct interaction experiments

For co-immunoprecipitation 25 μl of anti-HA resin (Ezview Red Anti-HA Affinity gel from Sigma-Aldrich) was used according to the manufacturer instructions. Briefly, cells were lysed with RIPA buffer (R078 from Sigma-Aldrich) and lysates were incubated with the anti-HA resin for 1 h at 4 °C on a rotating wheel. Co-immunoprecipitation of endogenous proteins was performed in HeLa cells using a crosslink immunoprecipitation kit (Pierce) following manufacturer instructions. Briefly, mouse anti-Rab7a antibody (Sigma) or mouse IgG was crosslinked to a resin using disuccinimidyl suberate (DSS) and incubated with pre-cleared HeLa cell lysate. After washing and elution immunoprecipitates were subjected to western blotting analysis using rabbit anti-Rab7a and anti-vimentin antibodies.

Immunoprecipitated samples were then loaded on SDS-PAGE and analyzed by Western blotting.

For pull-down His-tagged Rab7a wt and mutant proteins were expressed and purified from *Escherichia coli* BL21 as previously described [38]. 20 μg of each purified protein was bound to NiNTA resin at 4 °C for 45 min. Then the resin was washed 3 times for 5 min with a washing solution (NaH_2PO_4 50 mM, NaCl 300 mM, Imidazole 20 mM, pH 8.0) and incubated with the cellular lysate of N2A cells at 4 °C for 1 h. After extensive washing pulled-down samples were loaded on SDS-PAGE and analyzed by Western blotting.

For direct interaction GST, GST-tagged and His-tagged proteins were expressed in bacteria and affinity purified as described [17,38]. His-tagged Rab7a wt was incubated alone or in combination with GST or GST-tagged vimentin in PBS with 2 mM $MgCl_2$ and GTP 0.8 mM for 1 h on a rotating wheel. Subsequently, samples were subject to GST pull-down using a glutathione resin. Samples were then subjected to SDS-PAGE and Western blotting.

2.6. Antibodies

Mouse monoclonal 9E10 anti-Myc (ab32) and rabbit polyclonal anti-HA (ab9110) antibodies were from Abcam (Cambridge, UK), rabbit polyclonal anti-Rab7a (R4779), mouse monoclonal anti-Rab7a (R8779) and mouse monoclonal anti-tubulin (T5168) were from Sigma-Aldrich (St. Louis, MO), while anti-vimentin (sc-6260) and anti-vimentin phospho Ser38 (sc-16673) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-vimentin phospho Ser55 (ab22651) antibody was from Abcam (Cambridge, UK). Primary antibodies were used at a 1:500–1:1000 unless otherwise indicated. Secondary antibodies conjugated with fluorochromes (used at 1:500 dilution) or HRP (used at 1:5000 dilution) were from Invitrogen (Milan, IT) or Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.7. Western blotting

Cells were lysed in RIPA buffer and lysates were loaded onto SDS-PAGE. Separated proteins were transferred onto PVDF membrane from Millipore (Milan, IT). When phosphorylation was monitored,