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Research Article

Plectin deficiency affects precursor formation and dynamics of vimentin networks

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ABSTRACT

Plectin is a typical cytolinker protein that connects intermediate filaments to the other cytoskeletal filament systems and anchors them at membrane-associated junctional sites. One of the most important binding partners of plectin in fibroblasts is the intermediate filament subunit protein vimentin. Previous studies have demonstrated that vimentin networks are highly dynamic structures whose assembly and disassembly is accomplished stepwise via several intermediates. The precursor forms as well as polymerized (filamentous) vimentin are found in the cells in a dynamic equilibrium characterized by the turnover of the subunits within the polymer and the movement of the smaller precursors. To examine whether plectin plays a role in intermediate filament dynamics, we studied vimentin filament formation in plectin-deficient compared to wild-type fibroblasts using GFP-tagged vimentin. Monitoring vimentin and plectin in spreading and dividing cells, we demonstrate that plectin is associated with vimentin from the early stages of assembly and is required for vimentin motility as well as for the stepwise formation of stable filaments. Furthermore, plectin prevents vimentin networks from complete disassembly during mitosis, facilitating the rebuilding of the intermediate filament network in daughter cells.

Introduction

As a typical cytolinker protein of very large size (Mr>500,000), plectin has a multimodular structure that consists of a long central rod domain flanked by two globular domains. The α-helical rod domain mediates self-interaction, while the globular domains harbor binding sites for intermediate filament (IF) and various other proteins [1,2]. Plectin serves as a molecular bridge connecting IFs to microtubules and microfilaments, and anchoring them to junctional complexes at the cell surfaces, the subplasma membrane skeleton, and the nucleus [3]. In its networking capacity, plectin confers structural stability and protects the cells against mechanical stress. In agreement with such a role, patients carrying mutations in the plectin gene suffer from skin and muscular disorders, typified as EBS-MD (epidermolysis bullosa simplex combined with muscular dystrophy) [for a review see Ref. 4]. A similar phenotype, severe blistering within the basal layer of the epidermis and heart and skeletal muscle damage, was observed after plectin gene ablation in the mouse [5]. Beyond its structural function as a cytolinker, plectin plays a role in dynamic cellular processes and as an IF-based multifaceted scaffolding protein it recruits a variety of signaling molecules to the cytoskeleton, among them the non-receptor tyrosine kinase Fer, energy-controlling AMP-activated protein kinase (AMPK), the PKCδ receptor RACK1, endothelial nitric oxide synthase (eNOS), and the cell cycle kinase Cdk1 [2,6]. Being involved in structural as well as dynamic aspects

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Abbreviations: Cdk1, cyclin dependent kinase 1; GFP, green fluorescent protein; IF, intermediate filament; NuMA, nuclear mitotic apparatus protein; Plk1, polo-like kinase 1

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of all three of the major cytoskeletal filament network systems [2,7, and unpublished data] plectin is likely to emerge as a central player in many cellular processes requiring cytoskeletal restructuring and reorganization.

IF proteins are capable of self-assembly forming semi-flexible filamentous polymers that are stable but not static. Actually, cellular IFs are highly dynamic structures showing continuous exchange between polymers and smaller subunits, albeit without complete disassembly into individual subunits. As a result, cytoplasmic IFs exist in different shapes and sizes. The high motility of the smaller non-filamentous precursors and the dynamic interconversion from one form to the other facilitates the changes in distribution and organization that IFs undergo during cell growth, polarization, differentiation, and mitosis. The dynamics of vimentin filaments, which make up the IF network of fibroblasts, have been meticulously described in a series of studies [reviewed in Refs. 8,9].

As a major vimentin- and general IF-binding protein plectin constitutes an important organizing element of IF network cytoarchitecture [10–12], and thus may as well play an important role in vimentin filament dynamics. This issue remained largely unaddressed so far, except for a study showing that a recombinant fragment containing plectin's C-terminal IF-binding site inhibits IF formation in vitro in a dose-dependent manner [13]. Furthermore, consistent with a possible involvement of the protein in IF dynamics, it has been shown that keratin networks in plectin-deficient cells were more susceptible to osmotic shock-induced retraction from peripheral areas, and their okadaic acid-induced disruption proceeded faster [11]. Similar observations were made with cells deficient in epiplakin, a cytolinker that is structurally related to plectin [14].

In this study, we have reexamined the dynamics of vimentin filament formation using plectin-deficient fibroblasts and green fluorescent protein (GFP)-tagged vimentin. Monitoring vimentin and plectin behavior in spreading and dividing cells, we demonstrate that plectin is associated with vimentin from the early stages of assembly and is required for vimentin motility as well as for the stepwise formation of stable IFs.

Materials and methods

Cell culture, transfection, trypsinization, and replating

All experiments done routinely were carried out with low passage immortalized mouse dermal fibroblasts and the key observations were confirmed with primary fibroblasts. Immortalized cells were derived from plectin$^{+/+}$/p53$^{-/-}$ (wild-type) and plectin$^{-/-}$/p53$^{-/-}$ (knockout) mice as previously described [15]. Primary cells were derived in a similar manner from plectin$^{+/+}$ and plectin$^{-/-}$ mice. Experiments were performed with cells from passages 8 to 15 after isolation. Cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Confluent cultures grown on plastic dishes were trypsinized, dispersed into culture medium, and the cells replated onto coverslips at densities of 10^4–10^5/cm^2 [16]. For live-cell observations, a cDNA construct (pMG3) encoding GFP-tagged vimentin was transfected into wild-type and plectin-null fibroblasts using Fugene (Roche Applied Science) following the instructions of the manufacturer. 48 h after transfection, cells were trypsinized and replated onto glass coverslips as above. Plasmid pMG3 was generated by subcloning mouse vimentin cDNA (a generous gift of P. Traub; plasmid pMC-V21) [13] into vector pEGFP-C1 (BD Biosciences Clontech).

Synchronization by double thymidine block

Cell synchronization was performed by double thymidine block followed by nocodazole treatment. For the first block, exponentially growing cells were incubated with 2 mM thymidine for 16 h. This was followed by a 9-h release in which cells were washed and incubated in fresh medium, and a second block for another 16 h. Cells were then washed and cultivated for an additional 24 h in growth medium containing 400 ng/ml nocodazole. Mitotic cells that rounded up and detached from the Petri dish were harvested by mechanical shake-off [17], washed thoroughly to remove nocodazole, replated on polylysine-coated glass coverslips and then incubated at 37 °C to allow for cell cycle progression. At different time intervals, cells were fixed in 4% formaldehyde and processed for immunofluorescence microscopy.

Immunofluorescence microscopy

Fibroblasts, plated on coverslips, were rinsed rapidly in PBS, and fixed in 100 mM Pipes, 2 mM EGTA, 1 mM $MgSO_4$, pH 6.9 (PEM), supplemented with 4% formaldehyde for 6 min at room temperature. After fixation, cells were permeabilized in 0.1% NP-40 in PEM for 3 min at room temperature, and then briefly washed in PEM for 3 min. Rabbit anti-plectin antiserum #46 (diluted 1:500) [15], affinity-purified goat IgG to vimentin (diluted 1:1000) [18], and mAb to α-tubulin (diluted 1:1000; Sigma, B-512) were used as primary antibodies, and Cy5 donkey anti-rabbit IgG, Cy2-AffiniPure donkey anti-goat IgG, and Rhod Red-X-AffiniPure donkey anti-mouse IgG (all from Jackson ImmunoResearch) as secondary antibodies. Specimens were viewed in a Zeiss Axiophot fluorescence microscope and confocal images were obtained using an LSM 510 module (Carl Zeiss). Colocalization was evaluated by visual inspection of signal overlap on merged RGB confocal microscopy images (green and red channels showing vimentin and plectin stainings, respectively). Structures corresponding to squiggles or granules, displaying predominance of yellow pixels, were scored as colocalizing. For statistical evaluation of squiggle colocalization analyses we evaluated merged 8-bit images using the Colocalisation Highlighter plugin of open source software ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). Prior to colocalization analyses threshold settings for each 8-bit image (red and green respectively) were determined using the automatic thresholding function and assigned to the input window of the Colocalisation Highlighter plugin. The intensity ratio of colocalized pixels was set at 50%. Highlighted areas of maximal colocalization corresponding to elongated structures (squiggles), were scored as colocalizing.

Live-cell imaging and video analysis

Time-lapse video microscopy was implemented on a Zeiss Axiovert S100TV microscope equipped with phase-contrast and epi-illumination optics. Cells were spread on glass coverslips at a density of 2.8×10^5 cells/cm^2 and kept in phenol red-free DMEM during the whole period of observation. Cells were monitored in a closed POCmini cultivation system (Carl Zeiss MicroImaging, Inc) under 5% CO_2 and at 37 °C. Prior to recording, coverslips with cells