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The C-terminal domain controls the mobility of Crumbs 3 isoforms

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ABSTRACT

The physiological function of epithelia depends on an asymmetric distribution of their membrane domains. Polarity proteins play a crucial role for distribution processes, however, little is known about their mobility in epithelial cells. In this study, we analyzed the intracellular and plasma-membrane-associated mobility of fluorescence-labeled Crb3A and Crb3B. Both variants belong to the Crumbs protein family, which control size and identity of apical membranes in epithelial cells. Fluorescence recovery after photo-bleaching measurements revealed different mobilities for the two Crb3 variants. They also differentially affected mobility and localization of the Pals1/Mpp5 protein, which binds to Crb3A but not to Crb3B. In addition, tracking of intracellular vesicles indicated that Crb3A containing vesicles are slightly more immobile than Crb3B ones. Taken together, our data revealed different intracellular mobility patterns for Crb3A and Crb3B.

1. Introduction

Polarization of epithelial cells is achieved by the establishment of cellular contacts, such as tight and adherence junctions and the separation of the plasma membrane into an apical and basolateral domain. Each membrane domain has its own specific protein and lipid composition based on its functions including uptake, secretion, and *trans*-cellular transport [1–3]. During the last decade it was shown, that highly conserved CRB (Crumbs) and PAR (Partitioning defective) protein complexes are crucial for the establishment and maintenance of apical membranes whereas SCRIB (Scribble) polarity complexes control the formation of basolateral membrane domains [1–4].

The *crumbs* gene in the fruit fly *Drosophila melanogaster* regulates apico-basal polarity of epithelial cells and the formation of cellular junctions [5–7]. Mammalian organisms have three homologous genes of the *Drosophila crumbs* gene, called *Crb1*, *Crb2*, and *Crb3*. The Crb1 protein plays a pivotal role in the retina, since some mutations of this gene cause retinal diseases with progressive degeneration of photoreceptors, *e.g. Leber congenital amaurosis* (*LCA8*) and *retinitis pigmentosa* (*RP12*) [8–10]. Crb2 is expressed in retina, brain, and kidney [11]. Together with Crb1, Crb2 is crucial for retina development and maintenance [12–15]. In addition, Crb2 plays an essential role in early embryonic development, as mice lacking the Crb2 gene die at embryonic day 12.5 due to a strongly affected gastrulation [16]. In *Danio rerio* (zebrafish) the Crb2b protein is required for the establishment and maintenance of polarity in podocytes, which form a specialized epithelium at the renal filtration barrier [17]. This pivotal role of the *Crb2* gene in vertebrate kidneys was recently confirmed by two studies showing that mutations in the human *CRB2* gene are associated with congenital nephrotic syndrome [18,19]. The third mammalian *Crumbs* gene, *Crb3*, is highly expressed in epithelial tissues and is the predominant isoform in the kidney [20]. *Crb3* knockout mice show extensive defects in epithelial morphogenesis. Mice die shortly after birth, accompanied by cystic kidneys and proteinaceous debris throughout the lungs [21,22].

All *Crumbs* genes encode for single span transmembrane proteins. Similar to the Crumbs (Crb) protein of the fly, mammalian Crb1 and Crb2 isoforms possess large extracellular parts, which contain Laminin A/G and multiple EGF-like domains. In contrast, Crb3 is a short protein without characteristic protein domains [20]. However, all Crumbs proteins have a short, highly conserved intracellular region, which contains a FERM (from: *four point one*, *ezrin*, *radixin*, *moesin*) and a PDZ (from: *post synaptic density protein*, *disk large tumor suppressor*, *zonula*

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occludens-1) domain-binding motif. The FERM-domain binding module (FBM) mediates interactions to FERM-domain containing proteins which often serve as adaptors between the plasma membrane and the cytoskeleton [23–25]. Moreover, in the fly system it has been shown that Crb interaction with the FERM protein *Expanded* modulates Hippo signaling, which controls tissue growth and size in higher organisms [26–28].

The canonical PDZ-domain binding motif (PBM) mediates the interaction with Pals1, which is a component of the CRB-complex, and with Par6, which belongs to the PAR-complex [29–33]. Interestingly, alternative splicing of the fourth and final exon of the *Crb3* gene results in two different proteins called Crb3A and Crb3B [34]. The PBM of Crb3A contains the conserved "ERLI" motif. Crb3B has a divergent C-terminus ending with a putative PBM ("CLPI") variant. Thus, Crb3B is the only member of the Crumbs-family, which lacks the conserved ERLI-sequence [34].

In this study, we examined the mobility of fluorescence-protein tagged Crb3A, Crb3B, and Pals1 in stably transduced cell lines of immortalized kidney podocytes. Strikingly, fluorescence recovery after photo bleaching (FRAP) measurements revealed a higher mobility of Crb3B at the plasma membrane (PM) than Crb3A. Mutants of Crb3A FBM and PBM showed that both motifs influence lateral mobility. Moreover, Pals1 intracellular mobility is dramatically reduced when co-expressed with Crb3A, but not with Crb3B. Single particle tracking (SPT) measurements of fluorescence-protein tagged Crb3 variants revealed different intracellular mobility patterns for Crb3A and Crb3B.

2. Material and methods

2.1. Constructs and cloning

The coding sequence of human Crb3A was amplified from pRevTreGFP-Crb3A (kind gift from Ben Margolis, Ann Arbor). Crb3B, full length Pals1 and the cytoplasmic part of human Nephrin (amino acids 1077–1241) were amplified using a human cDNA library derived from AB8 podocytes [35]. The expression cassettes encoding GFP- or SNAP-tagged version of Crb3A, Crb3B and Pals1 were cloned into pCR4-TOPO or into pENTR-D/TOPO Vectors (Life Technologies) according to the manufacturer's instructions. The GFP-Crb3A insert was mutagenized to generate the following Crb3 point and deletion mutants: a FBM mutant "GFP-Crb3A FERMmut" in which the highly conserved GTYRPSSEE sequence (amino acids 91–99) was changed into GTARASSEA by alanine replacing of tyrosine (Y93A), proline (P95A) and glutamic acid (E99A) (Fig. 1B, arrows), a PBM mutant "GFP-Crb3AΔERLI", that lacks the last four amino acids (aa) encoding for the ERLI motif of Crb3A, and the "GFP-Crb3 basic" deletion mutant only containing the first five amino acids of the cytoplasmic tail (REKRQ). The inserts of the pCR-TOPO/pENTR-TOPO plasmids were used to generate the retroviral expression plasmids pQCXIP-GFP-Pals1, -GFP-Crb3A, -GFP-Crb3B, -SNAP-Crb3A, -SNAP-Crb3B, and the described -GFP-Crb3A mutants by standard cloning techniques using *Not*I/*Pac*I or *Asc*I/*Pac*I. For yeast co-transformation assays pENTR-Crb3A CT and pENTR-Crb3B CT, encoding the C-terminal parts of

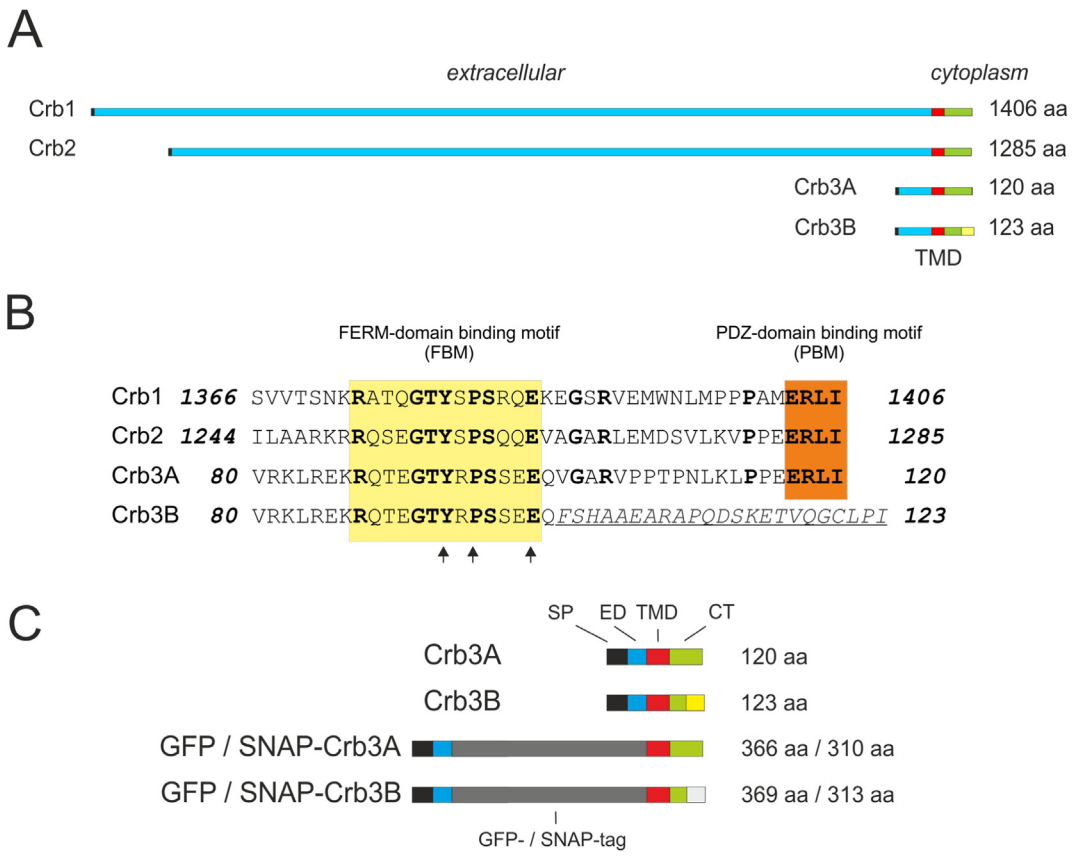

Fig. 1. Overview of Crb-family and design of Crb3 expression constructs. A: Schematic overview about the functional regions of mammalian Crumbs proteins. Crb1 and Crb2 contain large extracellular domains (blue), which is almost completely lacking in Crb3. All members of the Crumbs-family have a signal peptide (black) and one trans-membrane domain (red) followed by a short intracellular domain (green). B: Alignment of intracellular domains of the human Crumbs proteins, which consist of two main modules: the FERM domain binding (FBM) and the PDZ-domain binding motif (PDM). Alternative splicing of mammalian *CRB3* genes result in two different Crb3 proteins called Crb3A and Crb3B. Due to 23 different amino acids Crb3B lacks the ERLI motif (see also suppl. Fig. S1A). Arrows indicate amino acids which were changed into alanine in the used FERM binding domain mutant (Crb3A FERMmut). C: Scheme of Crb3A, Crb3B, and the GFP/SNAP-Crb3 fusion proteins that were used in this study. GFP- and SNAP-tags are integrated into the extracellular region of Crb3 isoforms (SP: signal peptide; ED: extracellular domain; TMD: trans-membrane domain; CT: C-terminal region).