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Interactions of cullin3/KCTD5 complexes with both cytoplasmic and nuclear proteins: Evidence for a role in protein stabilization

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ABSTRACT

Based on its specific interaction with cullin3 mediated by an N-terminal BTB/POZ homologous domain, KCTD5 has been proposed to function as substrate adapter for cullin3 based ubiquitin E3 ligases. In the present study we tried to validate this hypothesis through identification and characterization of additional KCTD5 interaction partners. For the replication protein MCM7, the zinc finger protein ZNF711 and FAM193B, a yet poorly characterized cytoplasmic protein, we could demonstrate specific interaction with KCTD5 both in yeast two-hybrid and co-precipitation studies in mammalian cells. Whereas trimeric complexes of cullin3 and KCTD5 with the respective KCTD5 binding partner were formed, KCTD5/cullin3 induced polyubiquitylation and/or proteasome-dependent degradation of these binding partners could not be demonstrated. On the contrary, KCTD5 or Cullin3 overexpression increased ZNF711 protein stability.

1. Introduction

As one of the 26 members of the KCTD protein family, KCTD5 is characterized by the presence of an amino-terminal BTB domain with similarity to the tetramerization domain (T1) of voltage-gated potassium channels. The BTB domain serves as protein interaction domain mediating the formation of both homomeric [1] and heteromeric [2] protein complexes.

Like other BTB proteins, different KCTD protein family members such as KCTD6 [3] and KCTD11 [4] have been characterized as substrate-specific adapters for cullin3-based ubiquitin E3 ligases [5,6]. The BTB domains of these adapter proteins mediate their interactions with cullin3 [5,6], whereas additional protein motifs such as zinc fingers (ZF), meprin and traf homology (MATH) or Kelch repeats are required for the binding of the respective substrate [2].

E3 ubiquitin ligases mediate the specificity of an enzymatic cascade responsible for both mono-and polyubiquitylation by recruitment of specific target proteins for the final step of the reaction [7]. Targeting proteins for proteasomal degradation is the main function of polyubiquitylation and involves linkage through internal ubiquitin lysine residue 48 [8,9]. In contrast, linkage through lysine residue 63 results in non-proteolytic regulation of substrate function [10]. Monoubiquitylationplays a role in regulation of histone activity, gene expression, DNA repair, endocytosis and protein sorting [11,12].

KCTD5 has been shown to interact specifically with cullin3, but not with other members of the cullin family [13]. On part of KCTD5, this interaction mainly requires the BTB domain [13], whereas on part of the cullin3 protein the N-terminal 75 amino acids already found to be important for the binding of confirmed substrate adapters [14] are required. These findings raised the possibility that KCTD5 also function as a substrate-specific adapter for cullin3-based ubiquitin E3 ligases. This hypothesis was addressed in the present study by searching for candidate KCTD5 interacting proteins, characterization of these interactions in mammalian cells and examination of a possible cullin3/KCTD5 mediated polyubiquitylation of these proteins.

2. Materials and methods

2.1. Plasmid constructs

All plasmid constructs were generated by standard restriction enzyme and PCR-based cloning techniques. pGBT9-KCTD5 contains

Abbreviations: AAV-2, adeno-associated virus type 2; BTB, bric-a-brac, tramtrak and broad complex; FAM193B, family with sequence similarity 193, member B; FOXO1, forkhead box O1; KCTD, Potassium channel tetramerization domain; MCM7, minichromosome maintenance protein 7; PAX3, paired box 3; ZF, zinc finger; ZNF711, zinc finger protein 711.

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the KCTD5 ORF derived from pCATCH-KCTD5 [15] in pGTB9 vector (Clontech). The complete ZNF711 ORF was amplified from HEK-293 cDNA and inserted into pCATCH vector [16] for expression of an N-terminal FLAG-tagged or pMyc-CS3+MT vector (Anne Vojtek, University of Michigan) for expression of a ZNF711 protein harboring 6 sequential N-terminal Myc-tags, respectively, both under control of the CMV promoter. The complete FAM193B ORF was assembled from PCR amplification of HeLa cDNA and one of the clones obtained in the two-hybrid screening and likewise cloned into pCATCH and pMyc-CS3+MT. The complete MCM7 ORF for cloning into pCATCH, pMyc-CS3+MT and a pCATCH derivate containing a 6 × His-tag was derived from pQBT7-MCM7 (Open Biosystems, MH51011-75176, MCM7, genbank accession number BC013375.2).

The pCATCH-KCTD5-47/234 and pCATCH-KCTD5-1/203 constructs have been described [15]. The additional KCTD5 deletion variants were generated by PCR amplification of the corresponding KCTD5 sequences.

The ubiquitin ORF and the cullin3 ORF for cloning into pMyc-CS3+MT vector were derived from PCR amplification of HeLa cDNA and plasmid expression clone IOH26262-pdEYFP-C1amp (imaGenes, Berlin, Germany), respectively.

2.2. Yeast two-hybrid screening

The full length KCTD5 ORF fused to the Gal4 DNA binding domain in the pGBT9 vector was used as a bait to screen a human kidney matchmaker® cDNA library from HEK-293 cells contained in the pACT2 vector (Clontech) by sequential transformation into yeast strain HF7c. Identification of positive transformants, isolation of library plasmids and elimination of false positives by retransformation with appropriate controls were performed as described in the manual for the matchmaker two-hybrid system 2 (Clontech).

2.3. Cell culture, transfection and drug treatment

Propagation and transfection of HeLa and HEK-293 cells were performed essentially as described [17]. Proteasome inhibitor MG132 (Sigma) was used at a final concentration of 3 μM for 16 h before harvesting, while protein synthesis inhibitor cycloheximide (CHX, Amresco) was applied at a final concentration of 10 μg/ml for the indicated time periods.

2.4. Cell synchronization and flow cytometry

For synchronization by a double thymidine block, HeLa cells were incubated 1 h post transfection with 2 mM thymidine for 18 h, followed by release into fresh medium for 9 h and a second treatment with 2 mM thymidine for 17 h. HeLa cells were collected at different time points after second release and subjected to western blot or immunofluorescence analysis. The percentage of synchronized cells was assayed by FACS analysis of DNA content after staining with 40 μg/ml propidium iodide (Sigma–Aldrich).

2.5. Immunoprecipitation and western blot analysis

Immunoprecipitation analysis was performed in RAF buffer as described [15] with anti-FLAG-M2 agarose (Sigma). Immunoprecipitates or whole cell extracts were subjected to western analysis as described [15].

2.6. Immunofluorescence

HeLa cells grown on coverslips were fixed and permeabilized as described [15]. Cells were incubated with primary antibodies at RT for 1–2 h, stained with FITC and/or TRITC conjugated secondary antibodies for 1 h and fixed with DAPI Fluoromount (Southern Biotech). The images were acquired either with Zeiss Axiophot 2 microscope with a Zeiss 63 × /1.4 NA Oil DIC objective or confocal microscope Zeiss LSM 510 META with a 63 × oil aperture objective.

2.7. Ubiquitylation assay

His-tagged proteins co-transfected into HEK-293 with N-terminally Myc-tagged ubiquitin were extracted and purified in NiNTA agarose (Qiagen) under denaturing condition as described [18], eluted in protein sample buffer complemented with 200 mM imidazole and analyzed by western blots.

3. Results and discussion

3.1. Determinants governing subcellular KCTD5 localization

Whereas the full-length KCTD5 protein of 234 amino acids displays a largely cytoplasmic localization [13,15], it can be translocated into the nucleus by the viral AAV-2 Rep78 protein [15]. With respect to potential cellular KCTD5 interaction partners, it was therefore of major interest to analyze the factors determining subcellular KCTD5 localization. With a comprehensive series of FLAG-tagged KCTD5 deletion variants (Fig. 1A) we could demonstrate that KCTD5 subcellular localization is influenced by its C-terminus in a complex manner (Fig. 1B). Deletion of 31 C-terminal amino acids (KCTD5-1/203) led to a nuclear localization of the corresponding protein variant. Deletion of additional 12 C-terminal amino acids (KCTD5-1/191) did not change this nuclear phenotype. Slightly larger deletions (KCTD5-1/181 and KCTD5-1/171), however, completely restored the cytoplasmic localization of the full-length protein. In contrast, a KCTD5 variant containing only amino acids 1 to 160 (KCTD5-1/160) showed a nuclear localization and deletion of the remaining C-terminal amino acids located outside the BTB domain (KCTD5-1/145) again switched the phenotype to a cytoplasmic localization. In addition to monomeric forms of the proteins, all the nuclear variants strikingly displayed a highly abundant band with reduced migration on western blots (Fig. 1C). The sizes of these additional bands were consistent with dimeric forms of the proteins stable under the conditions of SDS PAGE. By x-ray analysis, bacterially expressed KCTD5 has been shown to form pentamers [19] with the C-terminal 23 amino acids, which promoted cytoplasmic localization in our experiments, not visible on electron density maps and therefore predicted to be rather exposed to the outside. These and additional C-terminal sequences may thus regulate oligomere formation mediated by the BTB domain. The role of oligomerization in nuclear KCTD5 translocation was directly assessed by coexpression of the full-length protein together with the nuclear localized KCTD5-1/191 variant using two different tags for detection. In the presence of KCTD5-1/191, the full-length protein preferentially also was located in the nucleus in a high percentage of cells (Supplementary Fig. 1A). Coimmunoprecipitation analysis confirmed the formation of protein complexes between the full-length and the truncated protein (Supplementary Fig. 1B). Thus oligomerization most likely represents a prerequisite for nuclear translocation and not just a consequence of the later. A possible cell-cycle dependence of subcellular localization of the full-length KCTD5 protein was analyzed after HeLa cell synchronization through a double thymidine block (Fig. 1D) at several time points after cell release from G1/S into S phase of the cell cycle. During S phase, KCTD5 was predominantly located in the cytoplasm (Fig. 1E, t = 0 h and t = 3 h). An accumulation of KCTD5 in the perinuclear area was observed in the G2 phase at 6–7 h after release and at late stage M phase, KCTD5 was found

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