Interaction of Sedlin with chloride intracellular channel proteins

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Abstract Sedlin is an evolutionarily conserved protein encoded by the causative gene *SEDL* for spondyloepiphyseal dysplasia tarda. Nevertheless, how Sedlin mutations cause the disease remains unknown. Here, the intracellular chloride channel protein CLIC1 was shown to associate with Sedlin by yeast two-hybrid screening. Green fluorescence protein-CLIC1 readily co-immunoprecipitated with FLAG-Sedlin. In addition, both proteins colocalized extensively in cytoplasmic vesicular/reticular structures in COS-7 cells, suggesting their interaction at intracellular membranous organelles. Sedlin also associated with CLIC2 in yeast two-hybrid assays. The link between Sedlin and the intracellular chloride channels is the first step to understand their functional interplays.

Key words: CLIC1; Sedlin; TRAPP complex; Interaction

1. Introduction

The SEDL gene, which is mutated in spondyloepiphyseal dysplasia tarda, an X-linked recessive skeleton disease mainly characteristic of disproportionately short trunk and short stature, encodes a 140-amino acid protein named Sedlin [1,2]. Its mRNA is widely distributed in a variety of tissues, including heart, liver, and placenta. Sedlin is highly conserved from yeast to human [1]. Its ortholog in budding yeast, Trs20p, is a subunit of the transport protein particle (TRAPP) complex that plays key roles in vesicle docking/fusion processes during the endoplasmic reticulum (ER)-to-Golgi transport [3–5]. Recent studies indicate that this multiprotein complex is a nucleotide exchanger for Ypt1 and Ypt31/32 which are GTPases essential for the membrane trafficking [6,7]. How Sedlin functions in the TRAPP complex is currently unknown. In COS-7 cells, Sedlin localizes to the perinuclear region partly overlapped with the ER-to-Golgi intermediate compartment (ERGIC) [8]. Furthermore, a 2.4 Å resolution crystal structure of Sedlin reveals its similarity to those of the N-terminal regulatory domains of two SNAREs, Ykt6p and Sec22b, despite the lack of sequence homology to these proteins [9]. Sedlin is therefore speculated to exert regulatory and/or adapter functions in multiple protein–protein interactions in the ER-to-Golgi transport pathway. Nevertheless, how Sedlin mutations cause the rare osteochondrodysplasia is not known.

Sedlin also binds to MBP-1, a transcription repressor for the c-*myc* promoter [10]. Repression of c-*myc* expression by MBP-1 results in apoptosis. Binding of Sedlin appears to sequester MBP-1 in the cytoplasm, thus releasing its repression effects. These data indicate that Sedlin is also involved in cell growth regulation.

Five chloride intracellular channel proteins (CLICs) have been identified with wide distributions in membranes of, for instance, mitochondria, ER, and nucleus [11,12]. Their functions may cover homeostasis of cell membrane potential, substance transfer, and maintenance of intracellular pH and the size of cell volume. CLIC1 is the first member of the family identified in human [13]. It is a transmembrane protein sufficient to form a functional ion channel as a tetrameric assembly of subunits [14–17]. Considering their extensive homologies but distinct localization, different CLICs may function similarly at distinct locations [11,12].

To further investigate the functions of Sedlin in cells, we performed a yeast two-hybrid screening for Sedlin-associated proteins. Our results link Sedlin to intracellular chloride channels by demonstrating its interactions with CLIC1 and CLIC2.

2. Materials and methods

2.1. Constructs

The full-length Sedlin cDNA (kindly provided by Dr. Gengxi Hu) was cloned into pUHD30F [18] or pAS2-1 (Clontech) to express FLAG or Gal4 DNA-binding domain (BD) fusion protein. The FLAG epitope was located at the N-terminus of Sedlin. The fragment encoding the first 113 amino acids of Sedlin (SedlN) was also cloned into pAS2-1 after polymerase chain reaction (PCR). The full-length CLIC1 and CLIC2 were obtained by PCR using plasmid DNA from a placenta cDNA library (Clontech). The PCR fragments were then cloned into pACT2 or pEGFP-C1 (Clontech) to express Gal4 activation domain (AD) or green fluorescence protein (GFP) fusion proteins, respectively. Deletion mutant pACT2-CLIC1A encoding amino acids 138–241 was isolated from the yeast two-hybrid screening. pACT2-CLIC1B and pACT2-CLIC1C, encoding amino acids 138–215 and 138–201, respectively, were obtained by restriction cleavages (Fig. 1B). All clones derived by PCR were verified by sequencing.

2.2. Yeast two-hybrid screening

Two-hybrid screening was performed following the manufacturer's protocols (Clontech). Briefly, yeast Y190 cells were cotransformed with pAS2-Sedlin and a human placenta cDNA library constructed in pACT2 (Clontech). Positive clones on SD/-Leu/-Trp/-His/3-AT

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Abbreviations: AD, Gal4 activation domain; BD, Gal4 DNA-binding domain; CLIC, chloride intracellular channel protein; ER, endoplasmic reticulum; ERGIC, ER-to-Golgi intermediate compartment; β-gal, β-galactosidase; GFP, green fluorescence protein; TRAPP, transport protein particle

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agar medium were assayed for β-galactosidase (β-gal) activities using the lift-filter method. Plasmid DNA extracted from clones positive for β-gal was transformed into electrocompetent *Escherichia coli*. Plasmids from *E. coli* were again cotransformed with pAS2-Sedlin into Y190. Those still showing positive results were subjected to sequencing and further analysis.

2.3. Cell culture and transfection

COS-7 cells and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (BRL Gibco) supplemented with 10% calf serum (Sijiqing Company, Hangzhou, China) at 37°C in an atmosphere containing 5% CO_2. Transfection was performed using calcium phosphate method.

2.4. Coimmunoprecipitation

Co-immunoprecipitation was performed as described in [19]. Briefly, HEK293T cells growing on 100 mm Petri dishes were transfected with appropriate plasmids for 48 h. Cells were lysed in lysis buffer (50 mM HEPES, pH 7.8, 500 mM NaCl, 5 mM EDTA, 1% NP-40, 3 mM DTT, and protease inhibitors). After centrifugation, the supernatant was incubated with 25 μl 50% slurry of anti-FLAG M2 affinity resin (Sigma) for 1 h at 4°C. After washing three times (20 mM HEPES, pH 7.8, 250 mM KCl, 0.2 mM EDTA, 15% (v/v) glycerol, 0.1% NP-40, and protease inhibitors), the proteins were eluted with 25 μl of 0.8 mg/ml FLAG peptide. For immunoblotting, rabbit polyclonal anti-GFP antibody (Santa Cruz) was diluted 1:500 and anti-FLAG M2 mAb (Sigma) was diluted to 3 μg/ml.

2.5. Immunofluorescence microscopy

Cells grown on sterile coverslips were fixed in cold methanol prior to indirect immunofluorescence staining using appropriate antibodies. Rhodamine-labeled goat anti-mouse IgG antibody was from Pierce (Rockford, IL, USA). Images were captured with cooled CCD SPOT II (Diagnostic) on an Olympus BX51 microscope.

3. Results and discussion

3.1. CLIC1 as a candidate of Sedlin-associated proteins

In the yeast two-hybrid screening, 30 positive clones were collected for further analysis after screening of approximately 5×10^5 yeast clones. After further confirmation and partial sequencing, one of the positive clones, clone 60-1, was found to encode amino acids 138–241 of CLIC1 (named pACT2-CLIC1A). Since both Sedlin and CLIC protein are involved in functions of the intracellular membranous system and cell growth control [8,10,12,16,20], their interplay might be important for their functions and would provide further insights into the pathological roles of Sedlin in spondyloepiphyseal dysplasia tarda.

We therefore further tested their interactions using full-length CLIC1. Cotransformation of pACT2-CLIC1 with pAS2-Sedlin in yeast Y190 conferred β-gal expression (Fig. 1A, lane 3) and growth in medium lacking histidine (data not shown), suggesting that the full-length CLIC1 also interacts with Sedlin. Based on the crystal structure of Sedlin, it is suggested that the residues ^{111}MNPFY^{115} constitute a putative protein-binding motif [9]. We thus created a deletion mutant, SedlN, which lacked the C-terminal portion from residues 114–140. Indeed, the C-terminal 27 residues of Sedlin were critical for interaction with CLIC1 because co-expression of AD-CLIC1 with BD-SedlN failed to induce β-gal expression (Fig. 1A, lane 2).

Due to high similarities among members of the CLIC family [11,12], we tested if CLIC2 also bound to Sedlin. The full-length cDNA that we cloned coded for an extra 18 residues ('FCKDVPFTFFHDCEAFPA') (GenBank accession AY191592) following the 15th residue of the published sequence [21]. After BLAST search against the human genomic

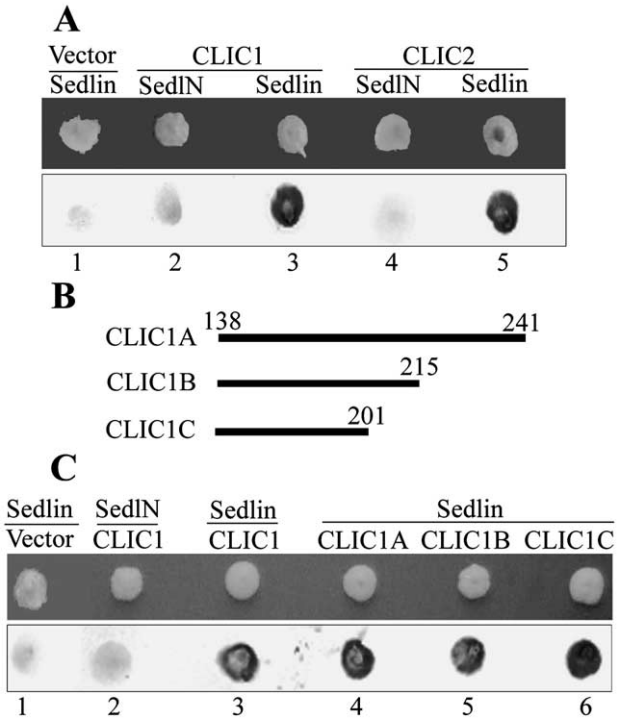

Fig. 1. Interactions of Sedlin with CLIC1 and CLIC2 in the yeast two-hybrid system. Yeast Y190 cells were transformed with appropriate plasmids to co-express a BD-Sedlin with an AD-CLIC as indicated. Colonies on SD/-Leu/-Trp medium were dotted on the same medium for an additional week and then photographed (upper lanes) prior to the lift-filter assays (lower lanes) for β-gal activity (dark). A: Interactions of CLIC1 or CLIC2 with Sedlin or SedlN. B: Diagrams of the CLIC1 deletion mutants. Numbers indicate positions of amino acids. C: Interactions of the CLIC1 mutants with Sedlin.

sequence, we found that this sequence was encoded by a novel exon located between exon 1 and exon 2 of the CLIC2 gene [21]. Similarly, CLIC2 also associated with Sedlin with similar sequence requirement for the latter in yeast (Fig. 1A, lanes 4 and 5).

To define the interaction region of CLIC1, we constructed an additional two CLIC1 deletion mutants, CLIC1B and CLIC1C, based on CLIC1A (Fig. 1B). Yeast two-hybrid assays indicated that the region between amino acids 138 and 201 was sufficient to bind Sedlin (Fig. 1B,C). Comparison with the sequences of other CLICs indicated that the binding region was highly conserved with 54% identity on average. It is possible that other CLICs also bind Sedlin using this region.

3.2. Interaction of CLIC1 with Sedlin in vivo

To determine whether CLIC1 associated with Sedlin in vivo, we performed co-immunoprecipitation. Due to lack of appropriate antibodies against endogenous Sedlin and CLIC1, FLAG-tagged Sedlin was transiently co-expressed with either GFP alone (Fig. 2, lane 1) or GFP-CLIC1 (lane 2) in HEK 293T cells. After immunoprecipitation with anti-FLAG M2 mAb, GFP-CLIC1 was readily detected by anti-GFP (Fig. 2, lane 4), while GFP alone was not (lane 3).

3.3. Colocalization of CLIC1 with Sedlin in COS-7 cells

We then tested if CLIC1 colocalized with Sedlin. In COS-7 cells, FLAG-Sedlin has been shown to locate at perinuclear

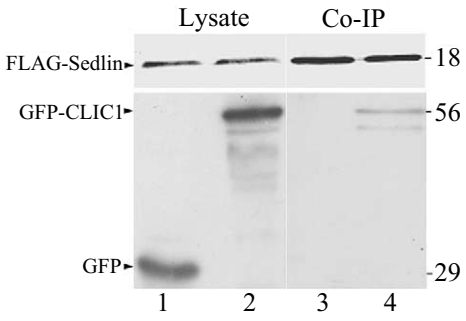

Fig. 2. Co-immunoprecipitation of CLIC1 with Sedlin. HEK293T cells were transiently transfected to co-express FLAG-Sedlin with GFP (lane 1) or GFP-CLIC1 (lane 2). After immunoprecipitation with anti-FLAG antibody, GFP-CLIC1 (lane 4) but not GFP alone (lane 3) was readily detected. The molecular mass (kDa) of each protein is labeled on the right.

regions partially colocalized with the ERGIC [8]. In our hands, although its perinuclear localization was visible, the protein also distributed throughout the cytoplasm as vesicular/reticular structures reminiscent of intracellular membrane networks (Fig. 3, panels 2 and 2a, 4 and 4a); some of them may be the ERGIC [8]. When GFP was co-expressed with FLAG-Sedlin (Fig. 3, panels 1 and 2), they both showed irrelevant patterns in the cytoplasm. This was easily judged with superimposed color images (data not shown), while gray-scale images at high magnifications were also helpful, a portion of which was shown (Fig. 3, panels 1a and 2a). On the other hand, although CLIC1 was originally identified as a nuclear chloride channel protein (NCC27) in CHO cells, it is not exclusively distributed in the nucleus [13]. Vesicular cytoplasmic staining is also detected [22]. Moreover, such a vesicular/reticular pattern in the cytoplasm is predominant in other cell lines, for instance, HeLa, PancI, and human macrophages [22]. Consistently, GFP-CLIC1 also exhibited vesicular/reticular distribution in the cytoplasm, in addition to strong localization at the perinuclear region and in the nucleus in COS-7 cells (Fig. 3, panel 3). In contrast to GFP, however, GFP-CLIC1 extensively colocalized with FLAG-Sedlin in the cytoplasm (Fig. 3, panels 3 and 4). Although their distribution patterns were not exactly identical, both proteins colocalized widely at the reticular, membranous organelle-like structures, as shown in the magnified images (Fig. 3, panels 3a and 4a). In addition to the cytoplasm, colocalization at perinuclear regions was also observed (Fig. 3, panels 3 and 4). These shared distribution patterns strongly suggest their interaction in intracellular membranous organelles.

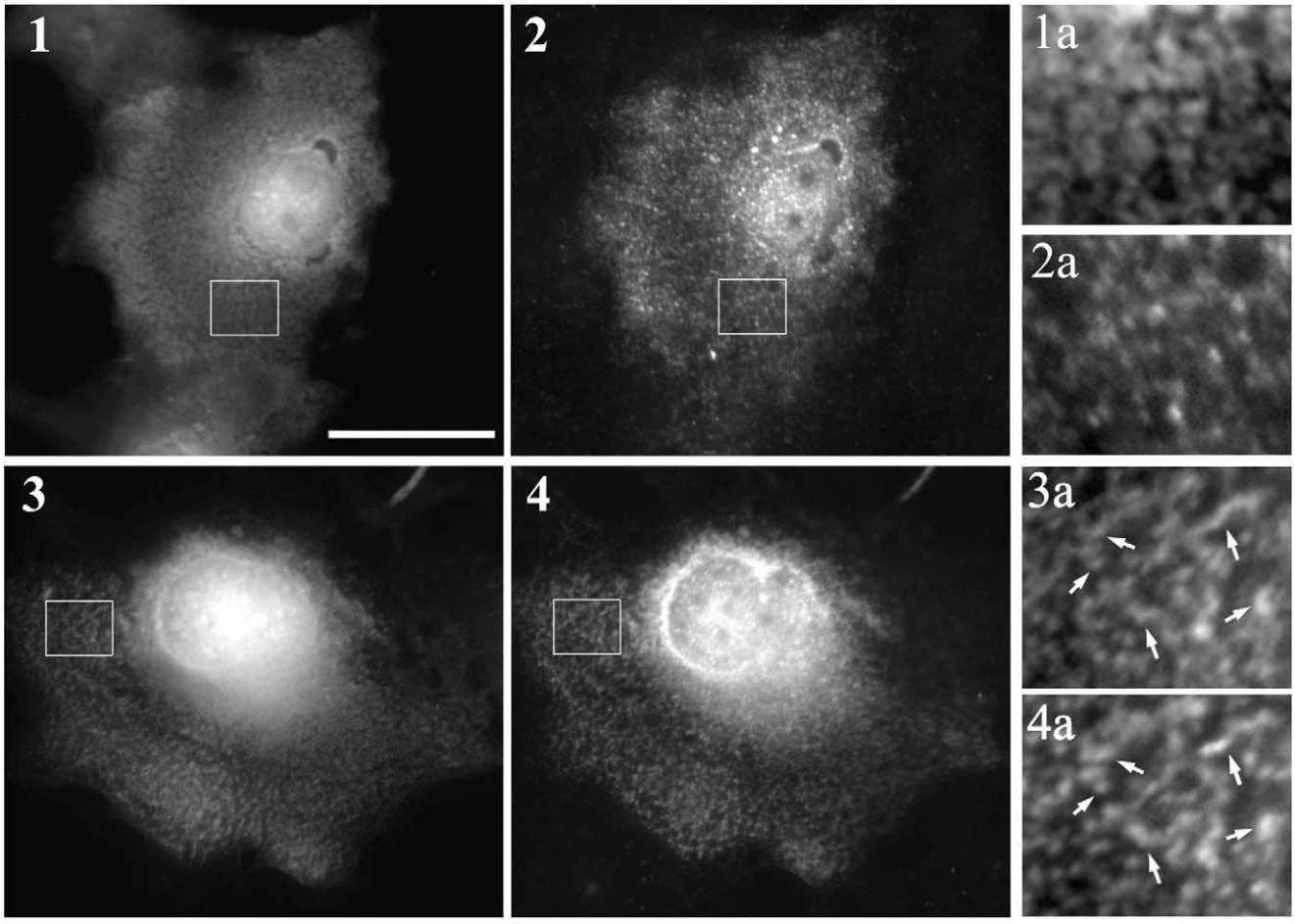

Fig. 3. Colocalization of CLIC1 with Sedlin. COS-7 cells were transfected to co-express GFP (panel 1) or GFP-CLIC1 (panel 3) with FLAG-Sedlin (panels 2 and 4). A representing area framed in each cell is further magnified four times with increased contrast to show details (panels 1a–4a). Arrows indicate typical regions where CLIC1 and Sedlin colocalize, although colocalization is not limited only to the indicated regions. Scale bar, 20 μm.

Taken together, our data demonstrate a clear interplay between Sedlin and CLIC1 in mammalian cells. Based on results for CLIC2, such interactions may be extended to other CLIC members. Since both Sedlin and CLIC proteins are membrane-associated proteins, their interactions might be important for either functions of the TRAPP complex or the proper targeting and functions of the intracellular chloride channels. This issue and whether such interaction is related to spondyloepiphyseal dysplasia tarda remain to be further explored in the future.

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