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Interaction of Sedlin with PAM14

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ABSTRACT

Sedlin is an evolutionarily conserved and ubiquitously expressed protein that is encoded by the gene SEDL. Mutations in the latter are known to be causative for spondyloepiphyseal dysplasia tarda. However, the mechanism underlying this remains unclear. We have previously shown that Sedlin interacts with the intracellular chloride channel proteins CLIC1 and CLIC2 in the cytoplasm. In this report we show that Sedlin is also physically associated with protein associated with MRG 14 kDa (PAM14), a nuclear protein that interacts with the transcription factor MORF4-related gene on chromosome 15 (MRG15). This was suggested by yeast two-hybrid screening and was confirmed with GST pull-down and immunoprecipitation assays. Moreover, we demonstrate that the C-terminus of Sedlin and the N-terminus of PAM14 are critical for their interaction. Together, these results suggest that nucleus-localized Sedlin may play a role in regulation of transcriptional activities of the MRG family of transcription factors via binding to PAM14. J. Cell. Biochem. 109: 1129–1133, 2010.

KEY WORDS: SEDLIN; PAM14; INTERACTION; NUCLEOLUS

Spondyloepiphyseal dysplasia tarda (SEDT) is an X-linked recessive skeletal disease characterized by a disproportionately short trunk, short stature, radiological features of the spine (posterior hump, end plate sclerosis, and disc space narrowing) and hips (short and thick femoral necks) in affected (hemizygous) adolescent males. Mutations in the gene SEDL encoding Sedlin are known to be causative for SEDT [Gedeon et al., 1999, 2001; Tiller et al., 2001].

Sedlin is a 16 kDa protein that is expressed in a variety of human tissues, including heart, liver, and placenta [Gedeon et al., 1999; Fan et al., 2003]. It is highly conserved from yeast to human, suggesting that it is likely to be critical for some fundamental cellular functions [Gedeon et al., 1999; Jang et al., 2002]. Sedlin has 41-amino acid residues identical to Trs20p, a subunit of the transport protein particle (TRAPP) complex in yeast [Sacher et al., 1998; Gedeon et al., 1999]. The TRAPP complex exists in two forms, TRAPPI and TRAPPII, both of which contain a Trs20p subunit [Sacher et al., 2001]. TRAPPI, as a guanine nucleotide exchange factor (GEF) for the Ypt1p GTPase, regulates the endoplasmic reticulum (ER)-to-Golgi protein transport. TRAPPII, as a GEF for Ypt31/32p, acts at the trans-Golgi network [Jones et al., 2000; Wang et al., 2000; Morozova et al., 2006]. Sedlin is therefore postulated to be involved in protein trafficking between the ER and the Golgi of cells [Gedeon et al., 1999, 2001; Tiller et al., 2001].

Sedlin has been reported to bind to MBP-1, a transcription repressor for the c-myc promoter [Ghosh et al., 2001]. Repression of c-myc expression by MBP-1 results in apoptosis [Ghosh et al., 2002]. Binding of Sedlin to MBP-1 appears to sequester MBP-1 in the cytoplasm, thus inhibiting its repressive effect on the transcriptional activity of c-myc [Ghosh et al., 2001]. These findings suggest that Sedlin may also contribute to regulation of cell survival and proliferation.

Human MORF4-related gene on chromosome 15 (MRG15) is a member of the MORF4/MRG family of novel transcription factors, which is known to play a role in regulation of cell proliferation and embryonic development [Bertram et al., 1999; Tominaga et al.,

Abbreviations: AD, Gal4 activation domain; BD, Gal4 DNA-binding domain; DAPI, 4′,6′-diamidino-2-phenylindole solution; DTT, dithiothreitol; PAM14, protein associated with MRG, 14 kDa; L-gal, L-galactosidase; GEF, guanine nucleotide exchange factor; GFP, green fluorescence protein; MRG15, MORF4-related gene on chromosome 15; SEDT, spondyloepiphyseal dysplasia tarda; TRAPP, transport protein particle.

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2005]. MRG15 interacts with Rb and is involved in the activation of the B-myb promoter under E2F control [Leung et al., 2001]. Nuclear protein associated with MRG 14 kDa (PAM14) has been identified as a MRG15-interacting protein, and has been reported to regulate Rb-induced E2F-responsive promoter repression, thus facilitating cell cycle progression [Leung et al., 2001].

We have previously shown that Sedlin interacts with intracellular chloride channel proteins 1 and 2 (CLIC1 and CLIC2), members of a novel CLIC family in the cytoplasm [Fan et al., 2003]. In this report, we demonstrate that Sedlin and PAM14 are physically associated in the nucleus, and the C-terminus of Sedlin and the N-terminus of PAM14 are required for this association. We propose that Sedlin may play a role in regulation of transcriptional activities of the MRG family of transcription factors.

MATERIALS AND METHODS

CELLS AND ANTIBODIES

COS-7 cells and HEK 293T cells were maintained in Hanks' minimum essential medium (Gibco) supplemented with 10% fetal bovine serum (Thermo Scientific Hyclone) at 37°C in an atmosphere containing 5% CO_2. The mouse monoclonal antibody against HA (F-7) and rabbit polyclonal antibodies against green fluorescence protein (GFP) were purchased from Santa Cruz Biotechnology, rabbit anti-human PAM14 (MRFAP1) polyclonal antibodies from Proteintech group, Inc., and mouse monoclonal antibody against Sedlin (TRAPPC2) (M01) from Abnova. Horseradish peroxidase (HRP)- and TRITC-conjugated secondary antibodies were from Promega.

PLASMIDS

The full-length Sedlin cDNA was cloned into pEGFP-C1 or pAS2-1 (Clontech) to express GFP or Gal4 DNA-binding domain (BD) fusion protein. The GFP tag was located at the N-terminus of Sedlin. The full-length PAM14 cDNA was cloned into pcDNA3.1 (Invitrogen) with N-terminal HA tag or C-terminal GFP tag. The fragment encoding the 1–85 and 86–127 amino acids of PAM14 was also cloned into pAS2-1 following polymerase chain reaction. Full-length Sedlin cDNA was also constructed into pGEX-3X (Amersham Pharmacia Biotech) with tag glutathione S-transferase. Transfection was carried out using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

YEAST TWO-HYBRID ANALYSIS

A yeast two-hybrid screen was performed using the Matchmaker two-hybrid system (Clontech). Briefly, yeast Y190 cells were cotransformed with pAS2-SEDL and a human placenta cDNA library constructed in pACT2 (Clontech). Positive clones on SD/-Leu/-Trp/-His/3-AT agar medium were assayed for L-galactosidase (L-gal) activities using the lift-filter method. Plasmid DNA extracted from clones positive for L-gal was transformed into electrocompetent *Escherichia coli* (strain TG1). Plasmids from TG1 were again cotransformed with pAS2-SEDL into Y190 cells. Those DNA inserts forming the positive clones were sequenced to determine the identities of the interacting clones.

GST PULL-DOWN ASSAY

The bacterial cell line BL21 (Stratagene) was transformed with the indicated plasmid. Expression of the GST fusion protein was induced by addition of isopropyl-D-galactoside for 3 h at 37°C. The bacterial lysates were subsequently harvested and purified on Sepharose 4B glutathione beads (Amersham Pharmacia Biotech). For GST pull-down assays with cell lysates, HEK 293T cells transiently transfected with the indicated expression plasmids were harvested in Nonidet P-40 lysis buffer (10 mM Tris–HCl, pH 7.4, 10 mM EDTA, 150 mM NaCl, 0.25% Nonidet P-40) supplemented with a protease inhibitor mixture (Calbiochem). Bead-immobilized GST-tagged proteins were incubated with the lysates for 3 h, washed four times in lysis buffer supplemented with a protease inhibitor mixture, and 1 mM DTT, solubilized in 2× sample buffer. The resulting proteins were subjected to Western blot analysis.

IMMUNOPRECIPITATIONS

Immunoprecipitation was performed as described previously [Fan et al., 2003]. Briefly, HEK 293T 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 inhibitor mixture). After centrifugation, the supernatant was pre-incubated with 3 μg of HA monoclonal antibody for 1 h at 4°C, followed by incubation with 25 μl of Protein A/G plus agarose beads (Santa Cruz Biotechnology) for 2 h at 4°C. After washing four times with washing buffer (20 mM HEPES, pH 7.8, 250 mM KCl, 0.2 mM EDTA, 15% (v/v) glycerol, 0.1% NP-40, and protease inhibitor mixture), the beads were boiled in 2× SDS buffer for 5 min, and subjected to SDS–PAGE and Western blot analysis. For the immunoprecipitation of endogenous proteins, cultured HEK 293T cells or Hela cells were directly lysed without plasmids transfected, and others were same as the above.

IMMUNOFLUORESCENCE MICROSCOPY

COS-7 cells grown on glass coverslips were transfected with GFP-Sedlin and HA-PAM14 plasmids. Cells were washed 24 h post-transfection and fixed with absolute methanol at 4°C for 2 min and washed with phosphate-buffered saline (PBS). Cells were incubated with anti-HA mouse monoclonal antibody (1:1,000) for 1 h at room temperature, washed, and then incubated with TRITC-conjugated goat anti-mouse IgG secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. After counterstaining with 4′,6′-diamidino-2-phenylindole (DAPI), coverslips were mounted onto glass slides with mounting medium (Dako) and examined using a fluorescence microscope (Zeiss, Axiovert+200).

PREPARATION OF NUCLEOLI

Enrichment of nucleoli was performed as described by Kieffer-Kwon et al. [2004]. Whole cell lysates were centrifuged at 4,000 rpm for 10 min in a microfuge. The cell pellet was resuspended in 3 ml of 0.25 M sucrose with 0.01 M $MgCl_2$ and layered on 3 ml of 0.35 M sucrose with 0.0005 M $MgCl_2$ and centrifuged for 5 min at 1,430*g* at 4°C. The pellet was then resuspended in 350 μl of 0.35 M sucrose with 0.0005 M $MgCl_2$ and pulse sonicated six times for 8 s. The resulting lysates were layered on 1.5 ml of 0.88 M sucrose with 0.0005 M $MgCl_2$ and centrifuged for 15 min at 5,000*g* at 4°C. The

pellet containing the enriched nucleoli fraction was dissolved in 2× SDS loading buffer and subjected to SDS–PAGE and Western-blotting analysis.

RESULTS

SEDLIN IS PHYSICALLY ASSOCIATED WITH PAM14

To better understand the physiological role of Sedlin in cells, we searched for novel Sedlin-binding partners by screening a human placental cDNA library with yeast two-hybrid assays using the full-length Sedlin gene as bait. Among positive colonies, one clone encodes the full-length cDNA sequence of PAM14. When Sedlin and PAM14 inserts were exchanged between DNA-AD and BD vectors, they remained associated with each other (data not shown). These results suggest that PAM14 is a candidate for Sedlin-binding proteins.

To confirm the interaction between Sedlin and PAM14, we performed GST pull-down assays on whole cell lysates from HEK293T cells transfected with PAM14-GFP using either GST-Sedlin or GST as bait. The proteins pulled down were subjected to Western blot analysis for Sedlin and PAM14. As shown in Figure 1, GST-Sedlin, but not GST, pulled down GFP-tagged PAM14.

To further verify that Sedlin and PAM14 interact with each other in the cell, we performed coimmunoprecipitation assays. HEK 293T cells were transiently cotransfected with HA-tagged PAM14 and GFP-tagged Sedlin. Whole cell lysates were subjected to immunoprecipitation with an anti-HA antibody. The resulting precipitates were analyzed by immunoblotting with anti-HA and anti-GFP antibodies. As shown in Figure 2A, GFP-Sedlin, but not GFP, was coprecipitated with the HA-PAM14 protein. Endogenous Sedlin and PAM14 were also associated in HEK 293T cells or HeLa cells (Fig. 2B). Taken together, these results indicate that Sedlin interacts with PAM14 within cells.

THE C-TERMINUS OF SEDLIN AND THE N-TERMINUS OF PAM14 ARE REQUIRED FOR THEIR INTERACTION

We have previously shown that the C-terminal 27 residues of Sedlin are critical for its interaction with CLIC1 and CLIC2 [Fan et al., 2003]. We therefore examined whether the same residues are required for binding of Sedlin to PAM14. As shown in Figure 3A, a deletion mutant of Sedlin, SedlN, which lacked the C-terminal residues 114–140, failed to interact with PAM14, suggesting that the C-terminal region of Sedlin is required for its binding to multiple partners.

To define the region of PAM14 that mediates its interaction with Sedlin, we constructed two PAM14 deletion mutants, PAM14N and PAM14C (Fig. 3B). Yeast two-hybrid assays showed that the

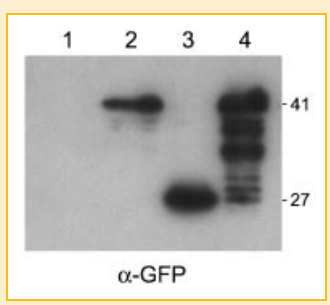

Fig. 1. The interaction of Sedlin and PAM14 by GST pull-down assay. PAM14-GFP lysate (lane 4) of HEK-293T cells was incubated with GST (lane 1) or GST-Sedlin (lane 2), lane 3: GFP lysate of HEK-293T cells. The molecular mass (kDa) of each protein is labeled on the right.

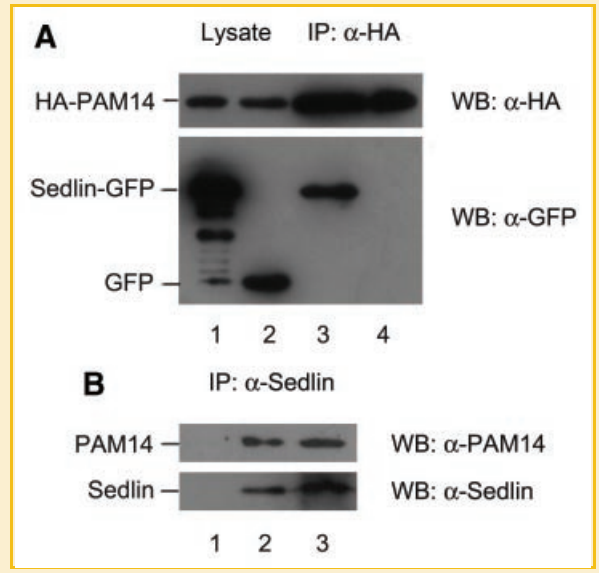

Fig. 2. The interaction of Sedlin with PAM14 by coimmunoprecipitation. A: HEK-293T cells were transiently transfected to coexpress HA-PAM14 with GFP (lane 2) or GFP-Sedlin (lane 1). After immunoprecipitation with anti-HA antibody, Sedlin-GFP (lane 3) but not GFP alone (lane 4) was readily detected. B: Lysates of HEK 293T cells (lane 2) and HeLa cells (lane 3) were immunoprecipitated by Sedlin antibody, and HEK 293T cells (lane 1) by mouse serum.

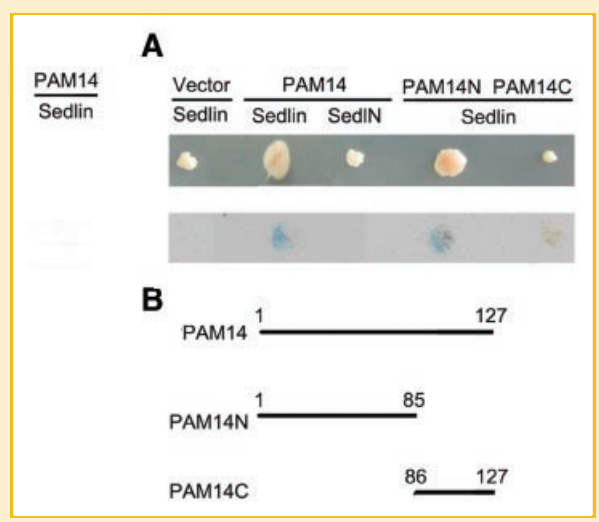

Fig. 3. The interaction of the Sedlin with PAM14 by yeast two-hybrid assays. A: Interactions of PAM14 with SedlN and PAM14 mutants with Sedlin in yeast Y190 cells. B: Diagrams of the PAM14 deletion mutants. Numbers indicate positions of amino acids.

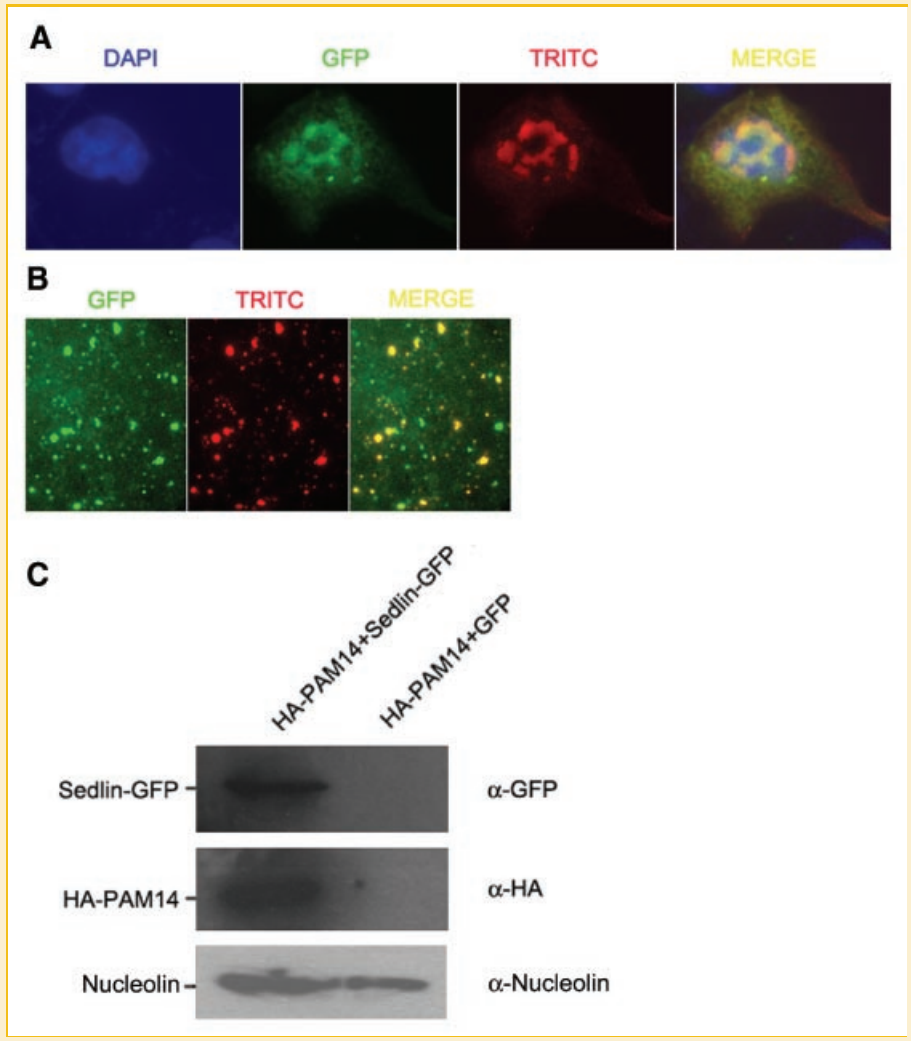

Fig. 4. The colocalization of PAM14 and Sedlin in cell nucleolus. Indicated cells were transfected to coexpress Sedlin-GFP with HA-PAM14 in nucleus (A) and nucleolus (B), and Western-blotting assay with nucleoli extracts (C). DAPI, GFP, and TRITC denote nucleus, Sedlin-GFP, and HA-PAM14, respectively.

N-terminal region (amino acid residues 1–85) of PAM14 is necessary for its binding to Sedlin (Fig. 3A), although the C-terminal region contains a predicted coiled-coil motif ranged from amino acid residues 92 to 126 (http://www.uniprot.org/uniprot), which is commonly involved in protein–protein interaction.

SEDLIN AND PAM14 COLOCATE TO THE NUCLEOLUS

We examined the subcellular colocalization of Sedlin and PAM14 using fluorescence microscopy in COS-7 cells cotransfected with GFP-Sedlin and HA-PAM14 as shown in Figure 4A. Both the Sedlin and PAM14 appeared to be localized in the nucleus (Fig. 4A). The same result was observed in HEK 293T cells (data not shown). To confirm the nuclear colocalization of the two proteins, nucleoli were extracted from the HEK 293T cells cotransfected with GFP-Sedlin and HA-PAM14, and were subjected to Western blot analysis. Figure 4B and C show that HA-PAM14 and GFP-Sedlin coexisted in nucleoli of the cells.

DISCUSSION

Mutations in the gene encoding Sedlin are known to be the cause of SEDT, an X-linked recessive skeletal disease [Gedeon et al., 1999]. However, the mechanism by which Sedlin mutations cause the disease remains unknown. To understand the biological role of Sedlin, we searched for novel Sedlin-binding partners. We demonstrate in this report that Sedlin interacts with PAM14, a binding partner for the transcription factor MRG15.

Our previous study has indicated the Sedlin is localized not only in the cytoplasm, but also in the nucleus of cells [Fan et al., 2003]. Indeed, Sedlin was found in this study to be associated with PAM14, a binding partner of the transcription factor MRG15, in the nucleus. Although PAM14 is not essential for normal mouse development and cell cycle regulation [Tominaga et al., 2004], MRG15 is known to block the Rb-induced repression of *B-myb*, leading to this promoter activation [Leung et al., 2001]. MRG15 contains helix–loop–helix and leucine zipper domains that are required for binding

to the N-terminus of PAM14 (N-terminal amino acids 1–50) [Leung et al., 2001; Zhang et al., 2006]. Sedlin also interacts with the N-terminal region of PAM14, suggesting that Sedlin may compete with MRG15 for binding to PAM14.

The nucleolus is the most prominent compartment in the nucleus, and it is the site for ribosomal RNA (rRNA) transcription, pre-rRNA processing, and ribosome subunit assembly [Lam et al., 2005]. High-throughput characterization of nucleolus proteins have revealed that the nucleolus contains cell-cycle proteins, kinases/phosphatases, DNA-binding proteins, and others, which are over 100 uncharacterized novel proteins [Andersen et al., 2005]. Localization of Sedlin in the nucleolus suggests that Sedlin may have a broad biological role in addition to its potential role in regulation of gene transcription by binding to PAM14.

The TRAPP complex mediates vesicle transport at different subcellular structures [Whyte and Munro, 2002]. Sedlin, as a component of TRAPP complex, has been reported to be involved in a defect in cartilage transport from the ER to the Golgi apparatus [Tiller et al., 2001], and is postulated to facilitate the assembly of the SNARE complex by displacing the regulatory SNARE domain and/or other proteins that interact with the regulatory domain [Sztul and Lupashin, 2006]. Recently, it has been shown that Sedlin is not involved in the regulation of Ypt1p, suggesting Sedlin is not required for TRAPPI functions [Kim et al., 2006]. In this paper, we report that Sedlin may play a novel role in cells.

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