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Up-regulation of PDCD4 in senescent human diploid fibroblasts

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Abstract

Programmed cell death 4 (PDCD4) has a common MI domain sharing with death associated protein 5 (DAP5) and a component of eukaryotic translation initiation factor (eIF4G) complex and it might also work as a tumor suppressor. We could find that the message and product of *Pdcd4* gene were up-regulated in senescent human diploid fibroblasts. In yeast two hybrid analysis, the C-terminal region of PDCD4 interacted with ribosomal protein S13 (RPS13), ribosomal protein L5 (RPL5), and TI-227H. In in vitro binding assay, RPS13, a component of 40S ribosome was stably bound to PDCD4. We also found that PDCD4 was localized to polysome fractions. We could pull out eIF4G with GST-PDCD4, but eIF4E did not interact with PDCD4. From these results, we could assume that PDCD4 might regulate the eIF4G-dependent translation through direct interactions with eIF4G and RPS13 in senescent fibroblasts.

Keywords: PDCD4; Senescence; EIF4G; Ribosomal proteins

Replicative senescence or cellular senescence is a phenomenon in which human diploid fibroblasts cultured in vitro undergo a finite number of cell divisions, after which they stop proliferating [1]. At this senescent stage, the cells may undergo many changes at the morphologic, biochemical, and molecular levels [2]. However, we do not have enough information on senescence-related molecular changes. In view of genetic hypothesis, the senescence-related genes like p53, p21, and telomerase actively regulated the aging process [3]. Using cDNA microarray, gene expression profile could be composed in various senescence conditions like replicative senescence [4–6], dietary restriction [7], and old rat brain [8,9].

The free radical theory of aging is the most popular and widely tested one for inducing DNA damage, protein modification, and lipid peroxidations [10]. Cellular changes of senescent cells would be due to repeated oxidative stress. In prolonged stress conditions like senescence, the protein translation profile has been found to be changed [11]. Recent work has shown that internal ribosome entry segments (IRESs) were utilized to maintain the protein expression under such stress conditions as mitosis, apoptosis, and senescence [12].

We screened human cDNAs to pick up the genes elevated in replicative senescence of human diploid fibroblasts. Among tens of genes related to cellular senescence, we gave our special attention to the genes involved only in senescent human diploid fibroblasts. Although PDCD4 contains a common motif, MI domain sharing with eIF4G, its function has not been clearly defined at the molecular level. From this study, we could prove that PDCD4 protein was elevated in cellular senescence and it worked through direct molecular interactions with eIF4G and ribosomal proteins.

Materials and methods

Cell culture. Human diploid fibroblasts (HDF) were isolated from Korean newborn foreskins and maintained as described previously [2]. HDF and HeLa cells were maintained in DMEM supplemented with fetal bovine serum and antibiotics.

Yeast two hybrid assay. C-terminal region of PDCD4 (amino acid 155–458) was subcloned into the *Eco*RI/*Xho*I sites of pDBD-GAL4

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"bait vector." Prey cDNAs from HeLa cells were cloned into the pAD-GAL4 vector. Screening was done as described in the manual (Clontech). We used SFY526 yeast strain and selected clones in triple dropout medium lacking leucine, tryptophan, and histidine. Protein–protein interactions were also confirmed by the X-Gal filter lift assay.

Purification of recombinant protein. pPDCD4-His6 construct was made by subcloning *PDCD4* gene into pET30a(+) vector (Novagen). pGST-RPS13, pGST-RPL5, and pGST-PDCD4 constructs were cloned in pGEX4T-1 (Pharmacia) using ribosomal protein S13, ribosomal protein L5, and PDCD4 cDNA, respectively. Recombinant proteins were raised from *Escherichia coli* BL21(DE3) and harvested by centrifugation and purified according to manufacturer's protocol.

Antibody and Western blot. Rabbit polyclonal anti-PDCD4 antibody was raised against recombinant PDCD4 protein by commercial service (KOMA Biotech, Korea). Total cell lysate was extracted using extraction buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% SDS) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrical transfer onto nitrocellulose filters (Protran, Schleicher, and Schulle). Immunoreactive bands were detected by each antibody and HRP-conjugated secondary antibody. We could visualize the corresponding proteins using an enhanced chemiluminescence detection kit (ECL Kit, Amersham).

Monoclonal anti-GST antibody, anti-actin antibody, polyclonal anti-eIF4G antibody, horseradish peroxidase (HRP)-conjugated anti-rabbit, anti-mouse, or anti-goat antibodies were purchased from Santa Cruz Biotechnologies.

GST pull-down assay. An in vitro protein–protein interaction assay was performed as previously described [13]. Briefly, glutathione–sepharose 4B beads containing a GST fusion protein were incubated with a pPDCD4-His6 in a total volume of 500 μl incubation buffer (20 mM Tris, pH 7.5, 75 mM KCl, 50 mM NaCl, 1 mM EDTA, 0.1% NP40, 10% glycerol, 1 mM DTT, and protease inhibitors (Roche)) for 1 h at room temperature. After centrifugation, the beads were washed four times with incubation buffer and interacting proteins were analyzed by Western blotting.

RT-PCR. The first strand cDNA was synthesized from 1 μg total RNA with reverse transcriptase and 1 μM oligo-dT primer. Each cDNA sample was amplified by PCR using specific primers against *PDCD4* (sense 5′-atggatgtagaaaatgagcag-3′ and antisense 5′-ttaaagtctt ctcaaatgccc-3′), *GAPDH* (sense 5′-ccacccatggcaaattccatggca-3′ and antisense 5′-tctagacggcaggtcaggtccacc-3′). Specifically amplified bands with correct sizes were analyzed by agarose gel electrophoresis and direct sequencing.

Polysome preparation. We prepared polysomal fractions, as described previously [14]. HeLa cells were treated with cycloheximide (100 μg/ml) for 5 min and then washed once with 20 ml phosphate-buffered saline containing cycloheximide and twice with 20 ml buffer A (5 mM Tris–HCl (pH 7.4), 1.5 mM KCl, 2.5 mM $MgCl_2$, 100 μg/ml cycloheximide). The cells were lysed in 400 μl buffer A containing 3 mM dithiothreitol, 30 U of RNasin (Promega), 0.5% Triton X-100, and 0.5% sodium deoxycholate. The lysates were centrifuged for 8 min at 4 °C at 3000*g*. The supernatant was collected and loaded onto 17–51% linear sucrose density gradients prepared in 20 mM Tris–HCl (pH 7.4)–80 mM NaCl–5 mM $MgCl_2$. The lysates were centrifuged at 4 °C for 2 h at 36,000 rpm in an SW41 rotor (Beckman). RNA was isolated from individual fractions by the proteinase K method.

Results and discussion

We screened the senescence-related genes using cDNA array containing 2500 human cDNAs (data not shown). Among them, we picked up tens of genes, the expression of which was significantly increased or decreased in senescent HDF. We were especially interested in *Pdcd4*, the function of which is not well defined yet.

First, we made polyclonal anti-PDCD4 antibody from rabbit by immunizing GST-PDCD4 fusion protein to examine the function of PDCD4. In immunoblot analysis using this polyclonal antibody, we could find the elevated level of PDCD4 protein in senescent HDFs (Fig. 1A). As we have already seen in the cDNA array, the transcript of *PDCD4* was increased in RT-PCR experiments (Fig. 1B). Although the expression of PDCD4 can be detected in differentiated cells [15], presenescent fibroblasts did not express PDCD4 at all. However, the levels of PDCD4 protein were significantly increased, when the cells were getting old.

The transcription of *Pdcd4* has been known to be regulated by the overexpression of phospholipase β3, which is frequently deleted in multiple endocrine tumors [16]. Differential expression of *PDCD4* gene may contribute to the suppression of multiple endocrine tumors. The expression of human *Pdcd4* was also decreased in promotion-resistant variants of mouse epidermal JB6 cells, whose PDCD4 was reported to inhibit neoplastic transformation in mouse JB6 cells [17,18]. The expression of PDCD4 was slightly up-regulated by NS-398, COX-2 inhibitor in HCA-7 cells [19]. Together with our data the expression of *Pdcd4* was inversely correlated with cellular transformation. Induction of *Pdcd4* in senescent cells would provide cellular resistance against the induction of tumorigenesis in vitro.

To find a clue for the function of PDCD4, we screened the interactions using yeast two hybrid system.

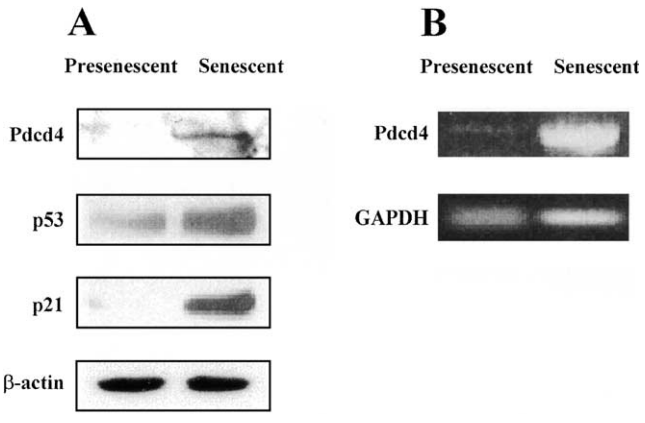

Fig. 1. Up-regulation of protein and mRNA of *Pdcd4* gene in senescent human diploid fibroblasts. (A) Protein extracts were prepared from presenescent and senescent fibroblasts and separated in SDS–PAGE. Immunoreactive bands to PDCD4 were detected in immunoblot using rabbit polyclonal anti-PDCD4 antibody. Other markers for senescence, p53 and p21, were also detected in senescent fibroblasts. (B) Total RNAs from each fibroblast were isolated and subjected to reverse transcription to synthesize first strand cDNA (SuperscriptII, Stratagene). Using specific primers for *Pdcd4* (sense 5′-atggatgtaga aaatgagcag-3′ and antisense 5′-ttaaagtcttctcaaatgccc-3′), *GAPDH* (sense 5′-ccacccatggcaaattccatggca-3′ and antisense 5′-tctagacggcag gtccacc-3′), each gene was amplified by PCR (ExTaq, Takara). Specific bands with corresponding sizes were analyzed by agarose gel electrophoresis.

We used C-terminal of PDCD4 as bait and HeLa cell cDNA library was the prey construct. After the selection of transformants in double or triple dropout media, we could select four clones (Table 1). Ribosomal protein P1 (RPLP1) was bound to PDCD4 only in triple dropout media assay. Ribosomal protein L5 (RPL5) and TI-227H of unknown function showed a weak interaction in β-galactosidase reporter assay. Ribosomal protein S13 (RPS13) showed a strong interaction with PDCD4 in several selection experiments. The major proportion of binding partners of PDCD4 was the ribosomal protein. To examine the molecular interactions further, we prepared fusion proteins, GST-RPS13, GST-RPL5, and PDCD4-His6. As shown in Fig. 2A, PDCD4-His6 was bound only to GST-RPS13 in an in vitro binding assay. But GST-RPL5, a component of 60S subunit of ribosome, did not form a complex with PDCD4 (Fig. 2B). Altogether with yeast two hybrid data, RPS13, a component of 40S subunit of ribosome should be a binding partner of PDCD4 in vitro as well as in vivo.

Table 1
Interaction of PDCD4 with human HeLa cell cDNA library in the yeast two hybrid system

Bait	Prey[a]	Function	TD[b]	X-gal[c]
PDCD4	RPLP1	Ribosome	+	–
	RPL5	Ribosome	+	+
	RPS13	Ribosome	+++	+++
	TI-227H	Unknown	+	+

[a] RPLP1, ribosomal protein P1; RPL5, ribosomal protein L5, RPS13, ribosomal protein S13.

[b] Survivial in triple dropout media: The interaction can be measured by the size and number of colonies in Trp–Leu–His-media. + (large), interaction; – (none), no interaction. The number of + signs represents the size of colonies.

[c] LacZ activity: The interaction can be semiquantitatively assessed by examining the blue color intensity of yeast colonies. + (blue), interaction; – (white), no interaction. The number of + signs represents the darkness of blue color.

Since PDCD4 was binding to the components of ribosome in yeast two hybrid experiment, we checked whether PDCD4 was present in the ribosomal complex. Polysomes were separated from cytosols in HeLa cells expressing PDCD4 by sucrose density gradient in the presence of RNase inhibitors. Although we could not

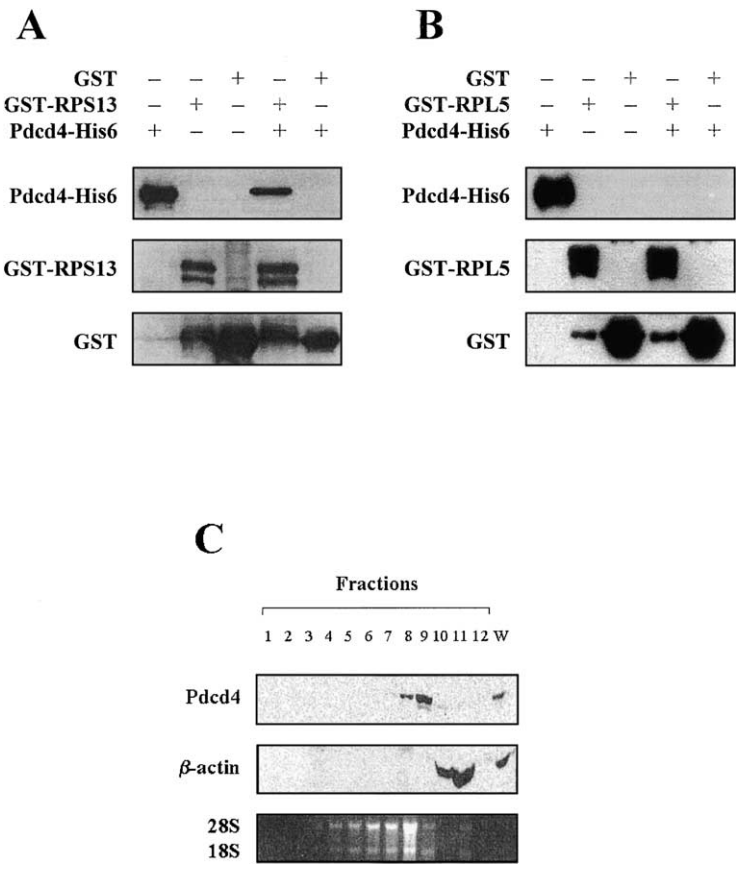

Fig. 2. Binding of PDCD4 with ribosomal proteins (RPS13 and RPL5) and PDCD4 localization in polysomal fractions. (A) Recombinant protein, GST- RPS13, was incubated with PDCD4-His6 and glutathione–sepharose 4B-bound complex was analyzed with antibodies against PDCD4 and GST. (B) After GST-RPL5 was incubated with PDCD4-His6, the glutathione–sepharose 4B-bound complex was analyzed by immunoblotting with antibodies against PDCD4 and GST. (C) HeLa cell extracts were separated by sucrose density gradient to isolate the polysome fraction and each fraction was analyzed with antibodies against PDCD4 and β-actin. Polysomal fractions can be assigned by examining the ribosomal RNA (bottom).

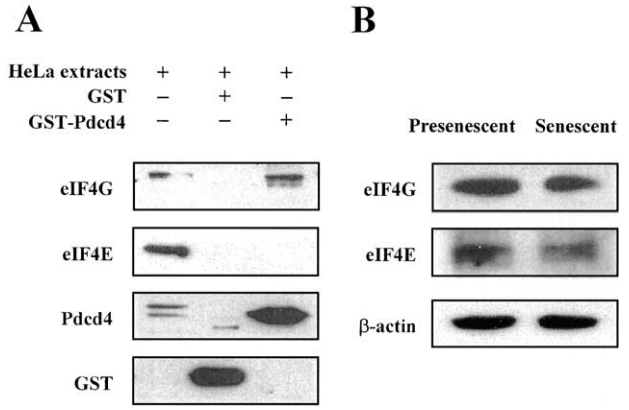

Fig. 3. Interaction of PDCD4 with eIF4G. (A) Total HeLa cell extracts were incubated with GST-PDCD4 for 1 h at room temperature. After separating the complex with glutathione–sepharose 4B bead, the binding complex was analyzed by immunoblot against eIF4G, eIF4E, and PDCD4. (B) The amount of eIF4G and eIF4E was examined in presenescent and senescent fibroblasts by immunoblot.

precisely fractionate polysome and monosome in this method, the fractions containing rRNA could be visualized by agarose gel electrophoresis (Fig. 2C). rRNA was enriched in the middle of fractions, in which PDCD4 was co-localized in Western blot. At the top of the gradient, β-actin was present which is the marker for free cytosolic fraction. From this pattern of distribution in sucrose gradient, we suggested that PDCD4 could be localized to ribosome complex. We need further experiments with a higher resolution to know about the interacting kinetics of PDCD4 protein in ribosomes.

Recently Aravind and Koonin [20] found conserved domains present in eukaryotic translation factors and in certain other proteins like DAP5 and PDCD4. The carboxyl termini of eIF4G and DAP5 shared a common motif with PDCD4, named MI after MA-3 (mouse homolog of PDCD4) and eIF4G. MI domain in these proteins provides a clue for the potential function in translation initiation or mRNA metabolism. eIF4G contained many motifs for interactions with other ribosomal machineries. We tried to check the interaction of PDCD4 with these complexes by the pull-down assay. GST-PDCD4 could pull out eIF4G from HeLa cell extracts in vitro (Fig. 3A). However, eIF4E was not detected by the GST-PDCD4 pull-down assay. The level of eIF4G or eIF4E content was not changed in senescent fibroblasts (Fig. 3B). Like DAP5, PDCD4 lacked the eIF4E-binding site. DAP5 is a caspase-activated translation factor, which mediates cap-independent translation at least from its own IRES [21]. We could speculate that MI motif in PDCD4 might confer the binding capacity with eIF4G. PDCD4 might replace the function of DAP5 in senescent cells, which conveyed resistance to apoptosis [22]. The putative function in apoptosis can be explained by the function of MA-3, the mouse homolog of PDCD4. This gene was originally cloned as an apoptosis-related gene, because it was first found in various apoptotic cells [23]. We need to show more direct evidence for the regulation of apoptosis by PDCD4 especially in senescence. From previous reports, several proteins related to cellular stresses, XIAP [24], Apaf-1 [25], and c-myc [26] would be the molecular targets of translational control by PDCD4.

Here we report the up-regulation of *Pdcd4* gene in senescent cells. Differential expression in senescent cells would be a good marker for senescence as well as for the resistance against transformation [16]. However, we need to further study the role of PDCD4 in aged individuals in vivo.

From these results, we suggest that the putative function of PDCD4 would be used as a component of eIF4G-dependent translation machinery. Molecular structure of PDCD4 had a signature of translational regulators. MI domain in PDCD4 could also be found in eIF4G and DAP5 [20]. In an effort to prove the precise function of PDCD4, we screened the counterpart of PDCD4 and found that it was localized to a complex with RPS13 and eIF4G. Although we need more direct evidence, we propose that PDCD4 might regulate the eIF4G-dependent translation regulation.

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