PDCD5 (human programmed cell death 5) plays a significant role in apoptotic and paraptotic cell deaths. However, it was found that recombinant PDCD5 added exogenously to culture medium could also enhance programmed cell death triggered by certain stimuli. Here we show that PDCD5 has a remarkable role in intercellular transport in various cells (endogenous caveolin-1-positive and -negative cells) through a clathrin-independent endocytic pathway that originates from heparan sulfate proteoglycan binding and lipid rafts. These conclusions are supported by the studies of slow internalization kinetics of PDCD5 endosomes, by the resistance of endosomes to nonionic detergents, by the overexpression of the clathrin dominant negative mutant form, which did not block PDCD5-fluorescein isothiocyanate uptake, and by PDCD5 localization in lipid rafts by immunofluorescence, electron microscopy techniques, and sucrose density centrifugation. This is further supported by the findings that certain drugs that disrupt lipid rafts, compete with cell membrane heparan sulfate proteoglycans, or block the caveolae pathway, impair the PDCD5 internalization process. The translocation activity of PDCD5 may possess physiological significance and be a potential mechanism for its programmed cell death-promoting activity. PDCD5 protein also has the ability to drive the internalization of large protein cargo, depending on the residues 109–115 mapped by deletion mutagenesis, and can translocate proteins is due to their ability to drive the internalization of large protein cargoes that are chemically coupled or fused to them. This group of proteins includes human immunodeficiency virus Tat (1, 2), Antennapedia (3), and herpes simplex virus VP22 (4), all of which are most commonly used in drug delivery and gene therapy studies. These translocatory proteins share several common features: they are released from cells by a pathway distinct from the recognized secretory routes involving a secretion signal (5, 6); they bind to target cells in a receptor-independent manner; and each of them has a highly basic region that appears to mediate the ability of these proteins to bind to polyanions, such as heparin/heparan sulfate (HS), polysialic acid, and nucleic acids. Deletion analyses of the first two proteins, Tat and Antennapedia, have mapped the apparent intercellular transfer function to the short runs of highly basic residues, termed protein transduction domains (PTDs), or cell-permeable peptides.

Early mechanistic studies showed that Tat-mediated transduction occurs through a rapid, temperature- and energy-independent process, suggesting direct penetration across the lipid bilayer. Because of the strong cell surface binding characteristics of the Tat PTD, measurements of protein internalization by flow cytometry or after fixation led to the above incorrect early assumptions regarding cellular uptake. Many studies have shown that Tat and Tat fusion proteins are rapidly internalized by lipid raft-dependent endocytosis or macropinocytosis after a cell surface interaction of Tat with cell membrane heparan sulfate proteoglycans (HSPGs) (7–14).

PDCD5 (programmed cell death 5), formerly designated as TFAR19 (TF-1 cell apoptosis-related gene 19), is cloned as a gene whose expression is increased during the apoptotic process of TF-1 cells induced by cytokine withdrawal using a cDNA-RDA method (cDNA representational differences analysis) (15). Previous studies have shown that PDCD5, when transiently overexpressed in TF-1, MGC-803, and HeLa cells, facilitates programmed cell death triggered by certain stimuli, such as growth factor withdrawal or serum withdrawal from culture medium (15) and enhances TAJ/TROY-induced paraptotic cell death (16). Moreover, PDCD5 translocated from...
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involves its reentry into the cells.

mote programmed cell death via a novel mechanism that involves transcellular delivery of various molecules. The translocation (11), human PDCD5 protein is a molecule similar to Tat. There- pathway and Tat involved in lipid rafts/caveolae endocytosis (26, 27). Our study shows that PDCD5 can enable transport across the cell membrane, resulting in human

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were all from Molecular Probes, Inc. (Eugene, OR). Protease

594-labeled cholera toxin subunit B and anti-EGFP antibody, TRITC- and FITC-labeled transferrin, and Alexa Fluor 633/Dy 800-conjugated anti-GFP antibody were obtained from Rockland. Secondary TRITC-labeled goat anti-mouse antibody, TRITC- and FITC-labeled transferrin, and Alexa Fluor 594-labeled cholera toxin subunit B and anti-EGFP antibody were all from Molecular Probes, Inc. (Eugene, OR). Protease

inhibitor mixture tablets were obtained from Roche Applied Science. Hoechst 33342 was from Sigma.

dsRed-Hub Plasmid Construction—The cDNA encoding Homo sapiens clathrin heavy chain residues 1073–1675 (28), clathrin hub fragment, was cloned into the BamHI and XhoI sites of the pDsRed-C3 vector (Clontech) to generate pDsRed-Hub for transfection. The plasmid was confirmed by DNA sequencing.

Cell Cultures and Transfection—HEK293 and U937 cells were maintained in RPMI 1640 (Invitrogen), and HT-29 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitro- gen) supplemented with 10% heat-inactivated fetal bovine serum (HyClone), penicillin (100 units/ml), streptomyycin (100 µg/ml), and l-glutamine (2 mM). 4 × 10⁶ HEK293 cells in 400 µl were transiently transfected by electroporation with 10 µg of the expression plasmid at 120 V for 20 ms, using an electric pulse generator (Electro Square Porator ECM 830, BTX, San Diego, CA). Cells were generally assayed 36–48 h after transfection.

Recombinant PDCD5 Protein—Recombinant PDCD5 protein was purified as described previously (15, 29). Briefly, Escherichia coli pop2136 harboring the prokaryotic expression vector pMTY4-PDCD5 was heated to induce the expression of the MS2-PDCD5 fusion protein. After denaturing, renaturing, and cleavage with thrombin, PDCD5 was purified by ion exchange chromatography with DEAE-Sepharose Fast Flow (Amersham Biosciences) and by gel filtration over Sephacryl S-200 HR (Amersham Biosciences). Fluorescein isothiocyanate (FITC) labeling of recombinant PDCD5 protein was prepared as described previously (30).

Subcloning and Deletion Mutagenesis—PDCD5 sequence was cloned into the EcoRI site of pEGFP-C3 vector (Clontech), and then EGFP and EGFP-PDCD5 sequence were cloned into the NotI site of pGEX-4T-2 vector (Amersham Biosciences) to generate pGEX-EGFP and pGEX-EGFP-PDCD5, respectively. To facilitate expression vector construction, a NotI recognition site was introduced at both ends of the open reading frame by PCR with primers. The C-terminal deletion mutants of PDCD5 (amino acids 1–115 and 1–108, respectively) were constructed using PCR amplification of the relevant portions of PDCD5 cDNA, followed by restriction digestion and subsequent subcloning into pGEX-4T-2 vector to generate pGEX-EGFP-PDCD5Δ116–125 and pGEX-EGFP-PDCD5Δ109–125, respectively. PDCD5 deletion constructs are designated PDCD5Δx-y, where x and y indicate the first and last deleted PDCD5 residue according to the complete human PDCD5 sequence (15). All constructs were sequenced using an ABI 3100 DNA sequencer.

The pGEX plasmids containing the required clone were transformed into the E. coli strain BL21 (DE3) to express the corresponding GST fusion proteins. GST fusion proteins were respectively bound to glutathione-Sepharose 4B resin (Amer- sham Biosciences). After on-column thrombin cleavage, the EGFP, EGFP-PDCD5, EGFP-PDCD5Δ116–125, and EGFP-PDCD5Δ109–125 proteins were released from GST, because the GST moiety remains bound to the Sepharose resin while the desired protein is eluted with PBS buffer.

p53N Peptide and p33N-PDCD5 Expression—The peptide ETFSDLWKLL from the Mdm-2 binding domain of p53, the cytoplasm to the nuclei and the up-regulated expression during apoptosis (17, 18) and the introduction of anti-PDCD5 antibody could suppress the etoposide-induced apoptotic effects of PDCD5 in HeLa cells (19). Recently, the decreased expression of PDCD5 has been reported in various human tumors, such as breast cancer (20), hepatocellular carcinoma (21), cervical cancer (22), gastric tumor (18), lung cancer (23), and chronic myelogenous leukemia (24). These observations suggest that PDCD5 plays a significant role in both apoptotic and nonapoptotic programmed cell death and may participate in the pathophysiologic course of diseases involving abnormal programmed cell death.

We have found that exogenously added human recombinant PDCD5 to culture medium of TF-1 cells or HL-60 cells can also enhance programmed cell death triggered by growth factor deprivation in TF-1 cells a or serum deprivation in HL-60 cells (25). An interesting question that remains to be addressed is how the exogenous PDCD5 enters the cells and promotes programmed cell death. Here we show that human recombinant PDCD5 protein makes use of clathrin-independent endocytosis to enter the cells. PDCD5 also has the ability to drive the internal- ization of large protein cargo EGFP that is fused to it. Fur- thermore, we have mapped a specific region of PDCD5 is necessary to drive translocation via mutagenesis assays. To determine whether the interaction of PDCD5 with the cell sur- face leads to the PDCD5 internalization, we tried to deliver a biologically functional peptide through PDCD5 into the cells. Others have found that the biologically functional peptide from the Mdm-2 binding domain of human p53, residues 17–26 (ETFSDLWKLL), fused to penetratin of the antennapedia to enable transport across the cell membrane, resulting in human cancer cell death (26, 27). Our study shows that PDCD5 can also introduce human p53 peptide into cells and induce their death. Compared with transferrin internalized by the clathrin pathway and Tat involved in lipid rafts/caveolae endocytosis (11), human PDCD5 protein is a molecule similar to Tat. There- fore, it may be useful for exploiting PDCD5 as a vehicle for transcellular delivery of various molecules. The translocation activity of PDCD5 also suggests that human PDCD5 may pro- mote programmed cell death via a novel mechanism that involves its reentry into the cells.

MATERIALS AND METHODS

Antibodies and Fluorescent Markers—Antibodies against clathrin heavy chain, transferrin receptor and cavelin-1 were from Transduction Laboratories (BD Biosciences). Anti-HSP70 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-histone H3 was obtained from Upstate Biotechnol- ogy, Inc. (Lake Placid, NY). IRDye 800- or IRDye 700-conju- gated anti-mouse and anti-rabbit IgG secondary antibody and IRDye 800-conjugated anti-GFP antibody were obtained from Rockland. Secondary TRITC-labeled goat anti-mouse anti- body, TRITC- and FITC-labeled transferrin, and Alexa Fluor 594-labeled cholera toxin subunit B and anti-EGFP antibody were all from Molecular Probes, Inc. (Eugene, OR). Protease

3 Y. Zhang and D. Ma, unpublished observations.
denoted as p53N, was synthesized by solid phase synthesis and purified by HPLC to 95% purity in GL Biochem (Shanghai) Ltd. The oligonucleotides encoding the residues 17–26 (ETFS-DLWKLL) from the amino-terminal of p53 were synthesized and subcloned into the pGEX-4T to express the p53N peptide fused to the N terminus of p53Mdm-2-binding previously described previously (30).

In vitro transcription of recombinant p53N-PDCD5 protein was prepared as one-Sepharose 4B resin (Amersham Biosciences). FITC labeling of recombinant p53N-PDCD5 protein was prepared as previously described previously (30).

Protein Subcellular Fractionation Analysis—5 × 10⁶ HEK293 cells or U937 cells were incubated with recombinant EGFP-PDCD5 for 5 h and washed in PBS, and cells were fractionated into cytosol, membranes, nuclei, and cytoskeleton using the Qproteome cell compartment kit from Qiagen according to the manufacturer’s instructions, and then one-fifth of each fraction was subjected to Western blotting. The fractions were analyzed to detect EGFP-PDCD5 protein and HSP70 and histone 3 proteins as the controls for cross-contamination during the cell fractionation procedure were sequentially reprobed with corresponding antibodies, respectively, on the same membrane.

Isolation of Triton X-100-insoluble Membranes—Isolation of Triton X-100-insoluble membranes was performed essentially as described previously (31, 32), with some modifications. 5 × 10⁶ HEK293 cells or 1 × 10⁷ U937 cells were incubated with recombinant EGFP-PDCD5 for 5 h, washed, and left in 1 ml of ice-cold 1% (v/v) Triton X-100 in MNE buffer (25 mM MES (pH 6.5), 150 mM NaCl, 5 mM EDTA, and 1× protease inhibitor mixture (Roche Applied Science)) and incubated on ice for 20 min. The cell suspension was then homogenized with a Dounce homogenizer at 20 strokes. The homogenate was then adjusted to 40% sucrose by the addition of 1 ml of 80% (w/v) sucrose in MNE buffer and loaded onto the bottom of a 5-ml ultracentrifuge tube. On top of this, 2 ml of 30% (w/v) sucrose in MNE buffer was overlaid, and then 1 ml of 5% (w/v) sucrose in MNE was overlaid. After centrifugation for 18 h at 100,000 g × 100,000 × g in a Beckman MLS50 swing-out rotor, 10 0.5-ml fractions were collected from the top of the gradient. To concentrate the raft fraction, each fraction was diluted 10-fold with ice-cold MNE and centrifuged in a Beckman MLS50 swing-out rotor at 150,000 g × 2 h at 4°C. The pellets were solubilized in sample buffer, separated by SDS-PAGE, and subjected to Western blotting. The same membrane was reprobed with corresponding antibodies, respectively.

Western Blotting—Proteins were transferred to nitrocelulose membranes (Hybond™ and ECL™; Amersham Biosciences). After blocking in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBS-T) and 5% (w/v) nonfat dry milk for 1 h at room temperature, membranes were incubated with IRDye 800-conjugated anti-GFP antibody or with corresponding primary antibody overnight at 4°C. Membranes were then washed with TBS-T three times for 10 min and directly detected or incubated with corresponding IRDye 800 or IRDye 700-labeled IgG secondary antibody in the dark for 1 h at room temperature. Following another three washes with TBS-T for 10 min, the membranes were scanned in the appropriate channels (800 nm for IRDye800 antibody, 700 nm for IRDye700 antibody) of the LI-COR Infrared Imaging System (Odyssey, Lincoln, NE) and analyzed with Odyssey software.

Drug Treatments—Cells were pretreated with the different drugs, 0.1–100 µg/ml heparin, 1.25–10 mM methyl-β-cyclo-dextrin (MβCD), 5 µM cytochalasin D, 20 µM nocodazole, 10–250 µM genistin, genistin, or 100 nM to 2.5 µM staurosporine, respectively, for 30 min in RPMI 1640 medium supplemented with 10% fetal bovine serum, after which time recombinant protein in fresh medium containing the same inhibitor was added. Cells were then processed after treatment with inhibitor and recombinant protein for fluorescence microscopy or flow cytometry. All these drugs were from Sigma.

Treatment with Triton X-100—Cells were incubated with recombinant PDCD5-FITC together with transferrin-TRITC for 5 h, washed, and left in ice-cold 1% Triton X-100 in PBS for 20 min before fixation with 2% paraformaldehyde.

Fluorescence Microscopy—For different fluorescent protein treatments, HEK293 cells were grown in specialized glass-bottom microwell dishes (MatTek Corp.) to about 50% confluence, and then fresh, 10% fetal bovine serum medium containing different fluorescent molecules was added. Final concentrations of fluorescent molecules were 1 µM recombinant PDCD5-FITC, p53N-PDCD5-FITC, Alexa Fluor 594-labeled cholera toxin B and/or transferrin-TRITC, or 1 µM protein of EGFP, EGFP-PDCD5, EGFP-PDCD5Δ116–125, or EGFP-PDCD5Δ109–125. After 5 h of treatment with recombinant proteins, cells were rinsed twice with PBS buffer and fixed with 2% paraformaldehyde in PBS for 15 min at room temperature. For immunostaining, fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min, washed and blocked with PBS containing 2% bovine serum albumin (BSA) for 30 min at room temperature, and incubated with a 1:200 dilution of primary antibodies in PBS supplemented with 2% BSA. Cells were rinsed three times with PBS and incubated with a 1:100 dilution of secondary antibodies for 30 min in 2% BSA in PBS. Cells were rinsed three times, all fluid was removed, and samples were mounted with 90% glycerin (Sigma) in PBS. For live cell recording, cells plated on 35-cm glass bottom dishes (MatTek Corp.) were placed in a humidified Plexiglas chamber and maintained at 37°C throughout the experiment. For the fast dynamics recording and co-localization experiments, cells were imaged using a TCS-SP laser-scanning confocal microscope with a ×40 or ×63 oil immersion lens (Leica Microsystems, Mannheim, Germany).

Electron Microscopy—To study the internalization of PDCD5 coupled to gold particles (PDCD5-gold), a pre-embedding procedure was performed. Briefly, according to the Colloidal Gold Conjugation Protocol (Schleicher & Schuell), PDCD5 gold labeling was done at pH 6.2 (for PDCD5, the pI is pH 5.9 (29)). HEK293 or HT-29 cells cultured on 12-well cell culture plates for 20 h were washed once with Dulbecco’s modified Eagle’s medium plus 25 mmol/liter HEPES containing 0.1% BSA at 8°C and then incubated for 1 h with PDCD5-gold (9 nm, 3.6 µg/ml) at 8°C and shifted to 37°C for 1 h to induce the internalization. After several washes, cells were scraped, pelleted for 5 min at 3000 rpm in an Eppendorf centrifuge, and fixed with 3% glutaraldehyde in PBS for 2 h at 4°C. Fixed cells were prepared for
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electron microscopy as previously described (33). Thin sections were cut, mounted on grids, and viewed in a JEM-1230 transmission electron microscope (JEOL, Japan) after contrasting with uranyl acetate.

**Flow Cytometry**—Quantification of internalized FITC-labeled PDCD5 recombinant protein was performed. Briefly, cells were plated in 24-well plates to about 70% confluence and incubated with 5 μg/ml recombinant PDCD5-FITC for the times indicated in Fig. 1, or cells were pretreated with the different concentrations of heparin (0.1–100 μg/ml) or the different concentrations of MβCD (1.25–10 mM) for 30 min and then incubated with 5 μg/ml PDCD5-FITC or p53N-PDCD5-FITC recombinant protein in fresh medium containing the same inhibitor. Cells were then washed, trypsinized, centrifuged, again washed twice with PBS, and finally analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).

**Programmed Cell Death Induction and Detection of Phosphatidylserine Externalization**—HT-29 cells were seeded into each well of 24-well tissue culture dishes containing 1 ml of culture medium. Cells were allowed to adhere for 24 h when the medium was replaced with media containing a 0.75–3 μM concentration of the recombinant p53N-PDCD5 protein, recombinant PDCD5 protein, or p53N peptide to be tested. Another set of wells (controls) in each experiment was processed identically but with peptide- and protein-free media. Cells were fed every 24 h with 0.3 ml of their respective peptide- or protein-containing media. The cultures were examined daily for changes in cell growth and morphology. Cells were released and collected after 2 days of treatment. 1 × 10⁶ washed cells were resuspended in 200 μl of binding buffer (PBS containing 1 mM calcium chloride). To detect phosphatidylserine (PS) externalization, FITC-conjugated annexin V (0.5 μg/ml final concentration) was added to the suspended cells according to the manufacturer’s instructions (Biosea, China). After incubation for 20 min at room temperature, 400 μl of binding buffer was added again, and samples were immediately analyzed on a FACSCalibur flow cytometer (BD Biosciences).

**Caspase-3-like Activity Assay**—Briefly, after treatment with a 3 μM concentration of the recombinant p53N-PDCD5 protein, recombinant PDCD5 protein, or p53N peptide for 2 days, incubation medium was removed, and the cells were lysed for 10 min in ice-cold lysis buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM Na₂HPO₄/NaH₂PO₄, 130 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride. Cell lysates were clarified by centrifugation at 18,000 × g for 20 min at 4 °C. Cell lysates containing 15 μg of protein were incubated at 37 °C in buffer containing 25 mM HEPES, pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.1% CHAPS, and 10 mM dithiothreitol with the fluorogenic substrate Ac-DEVD-7-amino-4-methylcoumarin (ALEXIS Biochemicals Industriestrasse). Fluorescence was measured with the use of a FLUOSTar fluorometer (BMG Labotechnologies) equipped with an excitation filter of 380 nm and emission filter of 460 nm. Results were calculated as a proportion of the control over 90 min (T₉₀/T₀). Samples were prepared in triplicate.

**RESULTS**

**Kinetics of PDCD5 Internalization**—The kinetics of cellular internalization of PDCD5-FITC in HEK293 cells and U937 cells is shown comparatively in Fig. 1. The PDCD5-FITC protein or FITC with the equal absorbance to PDCD5-FITC at 495 nm was added to the cell culture medium of human HEK293 or U937 cells, and cellular fluorescence was quantitatively assessed at different time points by flow cytometry. Analysis of the flow cytometry profiles and of the mean cellular fluorescence values shown in Fig. 1, A–D, clearly indicates that the PDCD5-FITC protein internalization kinetics in different cell types is similar. However, internalization of PDCD5-FITC was blocked at 4 °C in both cell types (Fig. 1G), suggesting that the internalization is an energy-dependent process. A microscopic analysis of cells treated with PDCD5-FITC protein indicated that fluorescence was localized to discrete compartments in the cytoplasm (Fig. 1E) with a very few labeled proteins reaching the nuclei (Fig. 1F), suggesting internalization by an endocytic process in this case.

**PDCD5 Is Not Internalized by Endocytosis in Clathrin-coated Endosomes**—To assess which endocytic pathway is involved in PDCD5 internalization, we mixed PDCD5-FITC to TRITC-labeled transferrin and added the two proteins to the culture medium of HEK293 cells.

It is known that transferrin is internalized from clathrin-coated invaginations on the plasma membrane that eventually detach to form clathrin-coated vesicles (34). As shown in Fig. 2A, PDCD5-FITC and transferrin-TRITC appeared in discrete intracellular endosomes; however, these compartments were clearly distinguishable. Transferrin-containing endosomes were dispersed through the cytoplasm and were enriched in the perinuclear recycling compartment, whereas PDCD5 endosomes were still localized in the cell periphery. It has been demonstrated that HEK293 cells express clathrin (35). Internalized PDCD5 does not co-localize with the clathrin (Fig. 2B) and transferrin receptor, a marker of the sorting/early and recycling endosomes (36) (Fig. 2C).

To avoid the fixation artifact, we added recombinant proteins PDCD5-FITC and transferrin-TRITC to live HEK293 cells and incubated the cells for 2 h at 37 °C. We analyzed internalization of PDCD5-FITC and transferrin-TRITC in living cells comparatively by time lapse confocal microscopy. Fig. 3 (also see supplemental Video 1) shows one confocal plane for each time point, with images taken for a total of 40 s of obser-
vations at 10-s intervals. In cells exposed to PDCD5-FITC and transferrin-TRITC, which appeared in clearly distinguishable compartments, the localization of the endosomes containing PDCD5-FITC was relatively immobile and in the cell periphery during this time frame, which is in sharp contrast to the relative mobility of transferrin-TRITC-containing endosomes. These results are in agreement with the notion that the velocity at which caveolar endocytosis proceeds is remarkably slower than that of clathrin-dependent endocytosis (11, 37).

A dominant-negative mutant form of clathrin is termed the hub fragment (28). The hub molecules interact with endogenous clathrin heavy and light chains and assemble into nonfunctional structures and disrupt clathrin-dependent functions. We transfected HEK293 cells with pDsRed-hub. The involvement of clathrin-coated endosomes in PDCD5 internalization was further ruled out by the observation that hubs did not block PDCD5-FITC uptake. In contrast, hub expression inhibited uptake of control FITC-conjugated transferrin (Fig. 2E). Taken together, these results clearly indicate that PDCD5 is not internalized by endocytosis in clathrin-coated endosomes.

**PDCD5 Internalization Occurs from Cell Membrane Lipid Rafts**—GM1 is commonly utilized as a marker for lipid rafts. Cholera toxin B is internalized by binding to ganglioside GM1 (38). Therefore, cholera toxin B can be used as a marker for lipid rafts. One group of cell membranes that have lipid raft properties are caveolae, typical flask-shaped membrane invaginations defined by a distinctive membrane coat composed of cholesterol-binding protein caveolin-1 (39). Caveolin-1 is a marker protein for caveolae. Therefore, we tested PDCD5-FITC co-localization with the labeled cholera toxin B and whether PDCD5-FITC endosomes were also positive for the presence of caveolin-1 endosomes. It has been demonstrated that HEK293 cells also express caveolin (35). As shown in Fig. 4A, in HEK293 cells, most PDCD5-FITC endosomes also contained the cholera toxin B, and a part of endosomes turned positive for both caveolin-1 and PDCD5-FITC (Fig. 4B), suggesting that PDCD5 was localized in caveolae and noncaveolar lipid rafts containing the ganglioside GM1.

To examine PDCD5 localization more directly, PDCD5 gold labeling and electron microscopy were employed. Caveolae were recognized by their characteristic size and shape. Remarkably, PDCD5-gold was associated with smooth and flask-shaped caveolae-like invaginations (Fig. 4C).

Lipid rafts are operationally defined by their insolubility in nonionic detergents. Thus, we tested the effect of Triton X-100 on HEK293 cells that had previously (2 h earlier) internalized both PDCD5-FITC and transferrin-TRITC. The detergent, ice-cold 1% Triton X-100, completely solubilized the transferrin endosomes, whereas endosomes containing PDCD5-FITC remained unaffected (shown in Fig. 5D). The internalization of fluorescent PDCD5-FITC in the presence of MβCD, a drug that extracts cholesterol from cell membranes, thus disrupting lipid rafts, in different cell lines was also quantitatively addressed by flow cytometry. PDCD5-FITC uptake in either HEK293 or U937 cells was blocked by treatment with MβCD, as show in Fig. 5, A–C. In Fig. 5A, in HEK293 cells, few internalized fluorescent endosomes of PDCD5-FITC were evident. In contrast,
internalization of transferring TRITC was kept, again indicating the involvement of lipid rafts in the PDCD5 internalization process.

Fig. 5D also shows representative cell images for each drug treatment. Cytochalasin D, a drug causing depolymerization of cell microfilaments, significantly impaired PDCD5-FITC internalization. In contrast, nocodazole, which disrupts polymerized microtubules, had no apparent effect on either PDCD5 or transferrin internalization. These observations support the idea that PDCD5 targets the endosomes that are connected with the actin microfilament cytoskeleton.

Furthermore, caveolae-mediated endocytosis has been shown to be regulated by the tyrosine kinase inhibitor genestin and the protein kinase C inhibitor staurosporine (40–43). As seen in Fig. 5E, PDCD5-FITC uptake is blocked by genestin and staurosporine, but not by genistin, the inactive 7-glucoside analogue of genistin, in HEK293 cells.

**PDCD5 Has the Ability to Drive the Internalization of Large Protein Cargo, and the C-terminal Region of PDCD5 Is Necessary to Drive Translocation**—As shown in Fig. 6, PDCD5 can deliver EGFP to cells when HEK293 cells were incubated with EGFP-PDCD5 fusion protein. To further corroborate the conclusion that the internalization of extracellular PDCD5 occurs from cell membrane lipid rafts, we did a biochemical fractionation analysis. Detergents, such as Triton X-100, are often employed for the separation of these molecular membrane complexes located in lipid rafts, using sucrose gradient centrifugation. When we isolated Triton X-100-insoluble membrane domains from the HEK293 cells incubated with EGFP-PDCD5 protein, comparing them with clathrin as a marker for Triton X-100-soluble membranes to ensure against incomplete solubilization of nonraft proteins, both EGFP-PDCD5 and caveolin-1 were found in the same light buoyant density fraction from sucrose gradient centrifugation of Triton X-100 cell lysates (i.e. in the Triton X-100-insoluble membrane domains) (Fig. 6A), suggesting both as raft proteins. EGFP-PDCD5 protein was enriched in low density Triton X-100-insoluble membrane domains also in U937 cells, although these cells do not express caveolin-1.

On the other hand, cells incubated with EGFP-PDCD5 protein were fractionated into cytosol, membranes, nuclei, and cytoskeleton using the Qproteome cell compartment kit from Qiagen according to the manufacturer’s instructions. As shown in Fig. 6B, EGFP-PDCD5 protein existed in each cell fraction compared with marker proteins specific to each fraction separated, consistent with the distribution shown in Fig. 6C by staining with an antibody specific for EGFP. Other studies have shown that endogenous PDCD5 can translocate to nucleus (17, 18). As shown in Figs. 6D and 1F, EGFP-PDCD5 or PDCD5-FITC can reach the discrete compartments in the cytoplasm and nuclei, but the fluorescence of EGFP-PDCD5 or PDCD5-FITC diffused into the cytosol and nuclei was hardly observed compared with Fig. 6C, which may be caused by the low brightness of fluorescence molecules diffused into the cytosol and nuclei or by EGFP unfolding and refolding in an optically active form only at very low efficiency during membrane translocation, as commonly occurs in this process (11, 44).

In order to delineate the region of PDCD5 that is essential for driving translocation, we expressed the sequential truncation mutants in *E. coli* and determined the internalization of each deletion (Fig. 7, A–C). EGFP-PDCD5Δ116–125 still is internalized. In contrast, EGFP-PDCD5Δ109–125 is almost not internalized. This finding suggests that the C-terminal region of PDCD5 (residues 109–115) might be important for the capability of PDCD5 to translocate through plasma membranes.

**PDCD5 Uptake Requires Cell Surface Heparan Sulfate Proteoglycans**—Observations reported previously indicate that the interaction between Tat and heparin/HS is specific and suggest a role for cell surface HSPGs in Tat uptake. A large body of evidence indicates that cell surface HSPGs are rapidly internalized through an endocytic pathway that may directly internalize ligands that bind to their glycosaminoglycan chains. Such an entry mechanism indicates that cell surface HSPGs have already been described for other HSPG ligands, including basic fibroblast growth factor, bacteria, and animal viruses (8, 45–47). To test whether this was the case for PDCD5 also, we studied PDCD5 interaction with HSPGs by treatment of cells with heparin.
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(a soluble competitor of cell membrane-associated HSPGs). A microscopic analysis of HEK293 cells treated with PDCD5-FITC and transferrin-TRITC indicated that heparin, impaired PDCD5 but not transferrin internalization (Fig. 8A). HEK293 and U937 cells were treated with different doses of heparin as indicated in Fig. 8B, incubated with PDCD5-FITC protein, and analyzed by flow cytometry to assess the amount of intracellular fluorescence. Cell treatment with heparin led to impairment of PDCD5 binding and internalization in a dose-dependent manner both in HEK293 and U937 cells.

PDCD5 Delivers p53N Peptide into Cells to Induce Cell Death—We attempted to deliver a biologically functional peptide into cells and elucidate if the interaction of PDCD5 with the cell surface leads to the PDCD5 internalization and thus test the usefulness of PDCD5 as a translocatory protein. As a functional peptide, we employed an Mdm-2 binding domain of human p53, ETFSDLWKLL (p53N), consisting of 10 amino acids. Drosophila Antennapedia homeobox domain, a PTD, has cell-penetrating capacity (3). p53N fused to the antennapedia PTD, penetratin, to facilitate cellular uptake, has been shown to induce cell death in human cancer cells (27).

We constructed and expressed a p53N peptide fused to the N-terminal of the PDCD5 (p53N-PDCD5) in E. coli. First, by confocal microscopy analysis, we observed PDCD5-FITC and p53N-PDCD5-FITC protein uptake in HT-29 colorectal tumor cells treated with 20 μg/ml PDCD5-FITC or p53N-PDCD5-FITC protein (Fig. 9A). Treatment of HT-29 cells with heparin or MβCD also led to impairment of PDCD5 and p53N-PDCD5 internalization in a dose-dependent manner, suggesting that PDCD5 and p53N-PDCD5 internalization involves lipid rafts and cell surface HSPGs in HT-29 cells (Fig. 9B and C).

We then incubated HT-29 cells with p53N peptide, PDCD5, and p53N-PDCD5 recombinant proteins for 2 days. A biochemical hallmark of programmed cell death is the translocation of PS from the cytoplasmic surface of the cell membrane to the external cell surface (48). Exposure of PS at the surface of apoptotic cells is easily determined by flow cytometry using fluorescence-labeled annexin V, which specifically binds PS (49). Fig. 9D shows the effects of treatment of HT-29 cells, with p53N peptide, PDCD5, or p53N-PDCD5 recombinant protein. p53N-PDCD5 (3 μM) recombinant protein evidently induces cell death in HT-29 cells as detected by annexin-V-positive cells using flow cytometry, but the treatment of cells with p53N peptide and PDCD5 recombinant protein had no effect, suggesting that PDCD5 functions as a translocatory protein, which mediates the intracellular delivery of the p53N peptide. Likewise, heparin can also block the cell death induced by p53N-PDCD5, indicating that p53N-PDCD5 binds to the HSPGs via PDCD5 prior to the internalization.

To further assess p53N-PDCD5 inducing cell apoptosis, caspase-3 activity was examined. Caspase-3, which can be activated by multiple pathways, is a key element in the signal cascade leading to programmed cell death. Fig. 9E shows measurements of caspase-3 activity in lysates prepared from HT-29 cells treated with a 3 μM concentration of the p53N-PDCD5 protein, PDCD5 protein, or p53N peptide or untreated control for 2 days. Caspase-3 activity in HT-29 cells was significantly increased when treated with p53N-PDCD5 protein compared...
with the untreated control, PDCD5 protein, or p53N peptide. Taken together, these data suggest that PDCD5 delivers p53N into cells to induce cell apoptosis.

**DISCUSSION**

Endocytosis encompasses distinct pathways, including clathrin-dependent and clathrin-independent endocytic processes. Clathrin-mediated endocytosis is one of the major mechanisms employed by cells for the receptors import from the plasma membrane into the cells. Clathrin-coated pits are involved in the internalization, subsequent degradation, and recycling of receptors participating in signal transduction events and nutrient import as well as in the reformation of synaptic vesicles. In contrast, lipid raft- and caveolae-mediated endocytosis represent important clathrin-independent endocytic pathways. They play important roles in processes such as

**FIGURE 5. Internalization of PDCD5 by lipid raft-dependent endocytosis.** A, cells were incubated with PDCD5-FITC and transferrin-TRITC in the presence or absence of 5 mM MβCD for 5 h and analyzed by confocal microscope. Note that internalization of PDCD5 was blocked by cholesterol depletion, whereas transferrin uptake was kept. B, as control, cells were incubated with PDCD5-FITC and Alexa Fluor 594-labeled cholera toxin B (Ctx B) containing the same MβCD for 5 h and analyzed by confocal microscope. Note that the internalizations of PDCD5 and cholera toxin B were both impaired by cholesterol depletion. C, cells were incubated with PDCD5-FITC containing the indicated concentrations of MβCD for 5 h and analyzed by flow cytometry (histograms indicate mean and S.D. of three independent experiments for the drug treatment). Note that internalization of recombinant PDCD5 was blocked by cholesterol depletion in a dose-dependent manner. D, PDCD5 uptake by HEK293 cells analyzed by confocal microscope after drug treatment. Note that PDCD5-FITC was found in detergent-resistant structures, whereas transferrin endosomes were disrupted by Triton X-100 extraction. Treatment with cytochalasin D (a microfilament-disrupting drug) impaired PDCD5 entry, whereas treatment with nocodazole (impinging on cell microtubules) did not affect PDCD5 uptake. Treatments with cytochalasin D or nocodazole did not block transferrin entry. E, HEK293 cells were incubated with PDCD5-FITC in the presence or absence of the indicated concentrations of genistein, genistin, or staurosporine for 5 h and analyzed by flow cytometry. Note that PDCD5-FITC uptake is blocked by genistein and staurosporine but not by genistin. Results are expressed as the mean with the S.E. indicated.
FIGURE 6. Intracellular delivery of EGFP by PDCD5. A. HEK293 cells or U937 cells were incubated with EGFP-PDCD5 for 5 h, homogenized, ultracentrifuged, and analyzed by Western blotting. The distribution of EGFP-PDCD5 was assayed with anti-GFP antibody, and caveolin-1 and clathrin were assayed with their specific antibodies by reprobing on the same membrane. Note that EGFP-PDCD5 and caveolin-1 were found in the same light buoyant density fraction (i.e. in the Triton X-100 insoluble membrane domains). B, the cells incubated with EGFP-PDCD5 were fractionated into cytosol (cyto), membranes (mem), nuclei (nuc), and cytoskeleton (cytoSk) using the Qproteome cell compartment kit from Qiagen according to the manufacturer’s instructions and analyzed by Western blotting. The fractions were analyzed to detect EGFP-PDCD5 protein, HSP70, and histone 3 protein, respectively, by sequential reprobings on the same membrane. C, HEK293 cells were incubated with EGFP-PDCD5 for 5 h, washed, fixed, and reacted with an anti-EGFP antibody followed by recognition with a rhodamine-labeled secondary antibody. In the same preparations, nuclei were also visualized by reactivity to Hoechst 33342. D, HEK293 cells were incubated with EGFP-PDCD5 for 5 h and fixed, and a six-image “z-series” was obtained by confocal microscope. Nuclei were visualized by reactivity to Hoechst 33342. Note that EGFP-PDCD5 protein can reach cytosol and nuclei.
cholesterol homeostasis and several types of signaling and favor
many different functions dictated by the actual demands of
the cell in relation to, for example, the stage of growth, differen-
tiation, or transformation.

Tat is the transactivator protein of human immunodeficiency
virus-1. Many studies have demonstrated that Tat
possesses the unusual property of entering the cells and trans-
locating to the nucleus when present in the extracellular envi-
ronment. The mechanism of entering the cells relates to the
interaction with the heparan sulfate chains of cell membrane
HSPGs and the active endocytic process originating from cell
membrane lipid rafts and involving caveolar transport (11).

This unusual characteristic depends upon the integrity of the
basic region of the protein, a nine-amino acid, arginine-rich
sequence that also corresponds to the nuclear localization sig-
nal and the trans-activation-responsive region binding domain of
the protein. This property is currently widely exploited as a biotechno-
logical tool for transcellular protein transduction.

In this paper, we have established
the first evidence that human
PDCD5 protein can enter various
cells by a clathrin-independent and
temperature-dependent endocytic
pathway that involves lipid rafts as
well as cell surface HSPGs like a
translocatory protein. This conclu-
sion is supported by the following.

(i) The kinetics of internalization
was relatively slow compared with
clathrin-dependent endocytosis.

When analyzed in a 10-s time scale,
PDCD5-containing endosomes in
living cells were relatively immobile
as compared with endosomes con-
taining transferrin, a marker of
cell-mediated endocytosis.

(ii) Using a clathrin dominant-negative
mutant inhibited uptake of control
FITC-conjugated transferrin but
did not block PDCD5-FITC uptake.

(iii) PDCD5 endosomes were resis-
tant to the nonionic detergent Triton
X-100, but transferrin endosomes
were not. Lipid rafts and caveolae
were all resistant to solubilization by
mild nonionic detergents such as
Triton X-100 at 4 °C.

(iv) PDCD5 internalization was selectively sen-
sitive to drugs that impair lipid raft
formation, such as MβCD. Lipid
rafts and caveolae are rich in choles-
terol and sphingolipid, so MβCD
extracts cholesterol from cell mem-
branes and disrupts lipid rafts and
caveolae. (v) Using immunofluores-
cence techniques determined the extensive co-localization of
PDCD5 proteins with cholera toxin B, a GM1-positive lipid raft
marker, in the cell periphery and the part with caveolin-1 in
cells expressing this protein but not with clathrin, transferrin
receptor, or transferrin. PDCD5-gold directly determined that
exogenous PDCD5 internalization was involved in the lipid
rafts/caveolae pathway. PDCD5 uptake was inhibited by the
tyrosine kinase inhibitor genestin and the protein kinase C
inhibitor staurosporine, which are two classes of inhibitors that
have emerged as relatively specific blockers of the caveolae
pathway in cells expressing caveolin-1. On the other hand, lipid
rafts and caveolae are connected with the actin microfilament

FIGURE 7. Deletion mutagenesis analysis of PDCD5. A, the schematic structure of EGFP fused with PDCD5
wild type and deletion mutants used in this study. PDCD5 has six exons. The C terminus of PDCD5 was trun-
cated. PDCD5 deletion constructs are designated PDCD5Δx–y, where x and y indicate the first and last deleted
PDCD5 residue according to the complete human PDCD5 sequence. B, purification analysis of recombinant
human EGFP-PDCD5 and the mutants by SDS-PAGE (12.5% acrylamide). Lane M, PageRuler prestained protein
ladder (Fermentas); lane 1, purified GST; lane 2, purified PDCD5; lane 3, purified EGFP-PDCD5; lane 4, purified
EGFP-PDCD5Δ116–125; lane 5, purified EGFP-PDCD5Δ109–125; lane 6, purified EGFP. C, cellular uptake of
EGFP, EGFP-PDCD5, and deletion mutants. Cells were incubated with 1 μM protein solution for 5 h at 37 °C and
were imaged using a TCS-SP laser-scanning confocal microscope. aa, amino acids.
or caveolae. These observations are similar to the reports on Tat endocytic pathway from other laboratories (11).

PDCD5 internalization occurs independently of caveolin-1 expression. We have observed that endocytosis of extracellular PDCD5 protein does not appear to be cell type-specific, since the protein is capable of entering every cell type so far tested, such as HEK293, HT-29, U937, and HL-60 cells (25) and HeLa, MGC-803, A549, PC-3, Jurkat T-lymphocytes, and TF-1 cells (data not shown). However, not all of these cells express caveolin-1 (52, 53). According to other findings, caveolin-1 does not act as a determinant of caveolae invagination and internalization but rather as a regulator that stabilizes caveolae at the plasma membrane (54). A recent review proposes that internalization via caveolae and internalization via lipid rafts are fundamentally similar processes (55). Caveolae are therefore a subdomain of the biochemically defined lipid rafts (56, 57). Cells where the caveolin-1 protein is not expressed do not develop caveolar invaginations; however, they have caveolar-equivalent plasma membrane domains (58). On the other hand, the lipid rafts in cells may be heterogeneous (59), and detergent-resistant membranes comprise at least two types of domains: caveolin-1-positive and not labeled for endogenous caveolin-1 or for transiently transfected caveolin-1-GFP (60–62).

**FIGURE 8. Internalization of PDCD5 in cells treated with heparin.** A, confocal microscope analysis in HEK293 cells incubated with PDCD5-FITC or transferrin-TRITC in the presence or absence of 10 μg/ml heparin. Note that heparin treatment can block PDCD5 but not transferrin entry. B, HEK293 and U937 cells were incubated with PDCD5-FITC in the presence or absence of the indicated concentrations of heparin for 5 h (HEK293) or 2 h (U937). HEK293 and U937 cells were analyzed by flow cytometry. The histograms indicate mean and S.D. of three independent experiments. Note that heparin treatment inhibited PDCD5 uptake.
According to our studies, PDCD5 internalization involves cell membrane lipid rafts extensively co-localized with cholera toxin B (a GM1-positive lipid raft marker) in the cell periphery and part co-localized with caveolin-1 in cells expressing this protein. Therefore, we speculate that extracellular PDCD5 protein enters various cells by non-caveolin-enriched lipid raft-mediated endocytosis and also simultaneously by caveolae-mediated endocytosis in endogenous caveolin-1-positive cells.

We found that PDCD5 had the ability to lead its EGFP fusion protein into the cells translocating to the cytoplasm and nucleus, which involved lipid rafts. Furthermore, the transduction with the fusion protein consisting of the PDCD5 and p53N peptide for death-inducing activity of p53N, the Mdm-2 binding domain of human p53, induced death in human cancer cells, indicating that biologically active peptide can be introduced into living cells by PDCD5. These results suggest that PDCD5 is a novel type of transloca-
PDCD5 Internalization Involves Lipid Rafts and HSPGs

Previous studies have shown that PDCD5 has six exons, and the exogenously added mutant of PDCD5 exon 6 deletion in cell medium lost the programmed cell death-promoting activity; in comparison, the exogenously added mutant of PDCD5 exon 1–2 deletion (63) and transiently transfected mutant of PDCD5 exon 6 deletion still kept their programmed cell death-promoting activity. Other studies on human PDCD5 structure using the heteronuclear nuclear magnetic resonance method indicate that there are a great number of positively charged and polar residues having high propensities for nucleic acid binding that can be found in PDCD5 segment Gln102–Lys115, and PDCD5 has a flexible unstructured C-terminal segment (64). Therefore, we speculate that C-terminal Gln102–Lys115 segment of PDCD5 represents cell-permeable peptide or PTD region. Furthermore, we designed two deletion mutants, EGFP-PDCD5Δ116–125 and EGFP-PDCD5Δ109–125. The former could be fully internalized, but the latter could not. This finding substantiated that the C-terminal region of PDCD5 (residues 109–115) should be important for the capability of PDCD5 to translocate through plasma membranes.

PDCD5 internalization originates from its binding with HSPGs. We have observed that PDCD5 interacts with the cell surface HSPGs in HEK293, U937, and HT-29 cells. Heparin also inhibited the cell death induced by p53N-PDCD5 through blocking of PDCD5 binding to the cell surface HSPGs and the consequent internalization. Many known translocatory proteins, such as Tat (8) and fibroblast growth factor (47), bind to the same surface molecules, HSPGs, before internalization, thus indicating that the internalization is dependent on these molecules. A common peptidic motif for heparin/HS binding consists of a region rich in basic amino acids flanked by hydrophobic residues (45, 65, 66). Studies on the importance of specific amino acids and regions of cell-permeable peptides or PTD secondary structure show that the highly basic region (Tat) or the central hydrophobic core (penetratin) is strictly required (67). Although PDCD5 has an isoelectric point of 5.5, it also contains highly basic regions. The C-terminal region of PDCD5 (residues 109–115) includes four basic residues, which may mediate the ability of the protein to bind to polyanions, such as heparin/HS, and translocate across membranes. Another study using capillary zone electrophoresis has determined that recombinant PDCD5 protein and synthesized PDCD5 peptide Val109–Val116 can interact with heparin.

Previous studies have shown that PDCD5 is an important regulator of both apoptotic and nonapoptotic programmed cell death (15, 16). We have found that heparin not only impairs PDCD5 protein binding to HSPGs and consequently leads to internalization but can also block programmed cell death-promoting activity by exogenously added PDCD5 protein to U937 cells (data not shown), suggesting that PDCD5 is endocytosed before being involved in signal transduction of programmed cell death pathways. The finding that many raft-associated proteins mediate signal transduction (68–70) implicates that rafts may concentrate PDCD5 with its interacting proteins as signaling platforms and facilitate the apoptotic and parapotic cell deaths when certain stimuli occur. The other studies have demonstrated that PDCD5 exists in sera from health donors as measured by ELISA analysis and that there was a higher PDCD5 level in sera of patients with systemic lupus erythematosus (71) or heart failure than that in healthy donors. These clinical assays coincide with a higher rate of cell death in these diseased states and PDCD5 up-regulation in response to various apoptotic stimuli (15). We speculate that after the startup of programmed cell death, the PDCD5 expression is up-regulated and can be directly released from dead cells and then reuptaken by other cells via lipid raft-dependent endocytosis to promote further programmed cell death. It may provide a novel mechanism for the positive regulation of programmed cell death via PDCD5 release from dead cells and consequent reuptake by other cells.

In conclusion, we have determined that PDCD5 protein is a novel, nonviral, and particular translocatory protein acting via the clathrin-independent endocytic pathway that involves lipid rafts as well as cell surface HSPGs, making it an attractive candidate for a novel cellular delivery system. We have testified that PDCD5 has the ability to lead biologically active peptide fused with PDCD5 into cells. Notably, PDCD5 is a phylogenetically conserved human protein expressed ubiquitously in various tissues with the ability to enter various cells, making it appropriate for a variety of therapeutic applications. Whereas Tat, Antennapedia, VP22, and many synthesized PTDs have been successfully used to import attached proteins, PDCD5 appears to be a novel and unusual delivery protein, which can serve as a tool for investigation of cell biology and biotechnological applications. In addition, PDCD5 release from cells to serum and its reuptake across the plasma membrane seem to reflect an in vivo biological process, which represents a potential mechanism for PDCD5 programmed cell death-promoting activity.

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