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Cytoskeletal Protein Vimentin Interacts With and Regulates Peroxisome Proliferator-Activated Receptor Gamma Via a Proteasomal Degradation Process

Yun-Chih Tsai,[1] Shu-Huei Tsai,[1] Emily Yun-Chia Chang,[1,2] Siow-Wey Hee,[1] Wei-Hao Chen,[2,3] Sheng-Chung Lee,[2] and Lee-Ming Chuang[1,2,4*]

[1]*Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan*
[2]*Institute of Molecular Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan*
[3]*Institute of Atomic and Molecular Sciences, Academia Sinica Taipei, Taiwan*
[4]*Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan*

ABSTRACT

Peroxisome proliferators-activated receptor gamma (PPARγ) receptor is a transcription factor that is located in and functions primarily in the nucleus. PPARγ is exported from the nucleus upon mitogen and ligand stimulation under certain circumstances. However, a cytoplasmic PPARγ interacting protein and its function have not been previously identified. Here, we report for the first time that cytosolic PPARγ interacts directly with cytoskeletal vimentin. We performed PPARγ immunoprecipitation followed by mass spectrometry to identify the vimentin-PPARγ complex. This interaction was confirmed by reciprocal vimentin and PPARγ immunoprecipitation and co-immunofluorescence examination. We demonstrated that PPARγ colocalized with vimentin in certain organelles that is golgi, mitochondria, and endoplasmic reticulum. In cells depleted of vimentin, PPARγ was ubiquitinated and targeted to a proteasomal degradation pathway. Together, these findings indicate a direct interaction of PPARγ with vimentin in the cytosolic compartment, in which vimentin appears to play a role in regulating the turnover rate of PPARγ, which may further regulate genomic or non-genomic activities through the regulation of PPARγ protein degradation. J. Cell. Biochem. 114: 1559–1567, 2013.

KEY WORDS: VIMENTIN; PPARγ; PROTEIN INTERACTION; PROTEIN DEGRADATION

Peroxisome proliferators-activated receptor gamma (PPARγ) is a ligand-dependent transcription factor and a member of the nuclear receptor (NR) superfamily that is essential for adipogenesis [Tontonoz and Spiegelman, 2008]. Two isoforms of PPARγ, PPARγ1 and PPARγ2, are generated by a combination of promoter usage and alternative splicing [Zhu et al., 1995]. PPARγ2 has an N-terminal extension of 30 amino acids and is expressed exclusively in adipocytes whereas PPARγ1 is expressed ubiquitously. As with all NR proteins, PPARγ consists of distinct functional domains including an N-terminal transactivation function-1 (AF-1) domain, a highly conserved DNA-binding domain followed by a hinge region, and a large C-terminal ligand-binding domain that contains a ligand-dependent transactivation (AF-2) domain.

The activity of PPARγ is well regulated and normally induced by binding of specific ligands that activate its gene transcription activity and initiate the expression of certain effector genes. High affinity synthetic ligands of PPARγ, thiazolidinediones, for example, rosiglitazone (BRL49653, BRL), have been used as antidiabetic drugs because of their effects on the regulation of lipid metabolism and their anti-inflammatory effects in adipose tissue [Lehmann et al., 1995; Yki-Jarvinen, 2004]. Several low affinity naturally occurring ligands, derived from long-chain fatty acids, including 9-hydroxyoctadecadienoic acid (HODE), 13-HODE found in oxidized low-density lipoprotein, 15-deoxy-Δ-12,14-prostaglandin (15d-PGJ2), and nitrolinoleic acids, are also able to activate PPARγ. More recently, we have demonstrated that 15-keto-

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*Correspondence to: Dr. Lee-Ming Chuang, Department of Internal Medicine, National Taiwan University Hospital, 7 Chung-Shan South Road, Taipei, Taiwan. E-mail: leeming@ntu.edu.tw

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prostaglandin E2 (15-keto-PGE2), a substrate of prostaglandin reductase 2, is also an endogenous ligand for PPARγ [Chou et al., 2007]. Transcriptional activity of PPARγ is regulated by the binding of specific ligands and by the recruitment of different coregulators (coactivators or corepressors) that work synergically to regulate gene expression through bindings to PPAR response elements in the genes. Upon binding of different ligands, differential interactions of PPARγ with certain cofactors, histones, and other transcription factors will take place and exert differential overlapping downstream gene activation/expression. Most of these PPARγ-interacting cofactors have been identified including coactivators such as CBP/P300, the SRC family, and TRAP220 [Powell et al., 2007] and corepressors such as SMART, NCoR, and RIP140 [Debevec et al., 2007].

Although the NRs are named for their prominent nuclear localization and genomic functions, evidence has been accumulating to demonstrate their extra-nuclear localization and non-genomic actions [Losel et al., 2003; Kampa and Castanas, 2006]. Although PPARγ has been found to reside and function mainly in the nucleus [Berger et al., 2000; Gurnell et al., 2000], there is also evidence for a significant cytosolic localization upon ligand binding [Thuillier et al., 1998; Shibuya et al., 2002; Varley et al., 2004]. Under various circumstances PPARγ is exported from the nucleus upon mitogen and ligand stimulation, and the shuttle is mediated by the nuclear export sequence in mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase 1/2 (MEK1/2) [Burgermeister et al., 2007]. This massive nuclear export reduces the ability of PPARγ to transactivate its target genes and thereby inhibits its genomic function. However, the fate of PPARγ proteins exported from the nucleus remains unknown, although some have suggested protein degradation [Burgermeister and Seger, 2007].

Here, we identified the intermediate filament (IF) vimentin as a cytosolic interacting protein of PPARγ using immunoprecipitation followed by mass spectrometric analyses. The domains of PPARγ, i.e., the AB and DEF domains, responsible for binding to vimentin, were also identified. We further found that upon depletion of the interacting protein, the turnover rate of PPARγ protein in 3T3-L1 cells was accelerated.

MATERIALS AND METHODS

CELL CULTURE

3T3-L1 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM; high glucose) plus 10% calf serum. Two days after confluence, differentiation was induced by the addition of DMEM containing 10% fetal bovine serum (FBS), 172 nM insulin (I), 1 μM dexamethasone (Dex, D), and 0.5 mM methylisobutylxanthine (Mix, M) for 4 days. The medium was then replaced with DMEM containing 10% FBS for full differentiation in 2–3 days. The effect of PPARγ ligand was assessed by inducing 3T3-L1 cells with either dexamethasone/insulin, with or without 0.5 μM BRL49653 or 10 μM 15-ketoPGE2. For adipogenesis assay, cells were then fixed and stained with Oil-Red O (Sigma) upon fully differentiated.

PREPARATION OF WHOLE CELL EXTRACTS AND SUBCELLULAR FRACTIONATION

Cell monolayers were washed with phosphate-buffered saline (PBS) and harvested in a lysis buffer containing 20 mM Tris, pH 8.0, 137 mM NaCl, 1 mM EGTA, 5 mM EDTA, 1% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and proteinase inhibitor mixture (Roche, Basel, Switzerland). Samples were extracted on ice for 30 min prior to centrifugation at 12,000 rpm for 30 min at 4°C. The resulting supernatants were analyzed for protein content by bicinchoninic acid (BCA) analysis (Pierce) and stored at −80°C until used. For preparation of four subcellular fractions, cell extracts were collected with Qproteome Cell Compartment kit (Qiagen) following the instructions. To further concentrate proteins, each fraction of extract was then subjected to acetone precipitation to remove salts and lipid soluble contaminants.

IMMUNOPRECIPITATION

After 3T3-L1 cells were fully differentiated, whole cell lysates and nuclear/cytosolic/membrane/cytoskeletal extracts were collected as described above.

For co-precipitation with PPARγ, 1 mg of pre-cleared cell lysate was incubated with 2 μg of anti-PPARγ-agarose conjugate (Santa Cruz Biotechnology) and the samples were rotated overnight at 4°C on a rotating platform. For immunoprecipitation with anti-vimentin and ubiquitin, 2 μg of each antibody was first incubated with 40 μl protein G-agarose beads for 1 h at 4°C. Then, 1 mg of pre-cleared cellular extracts was added to the antibody-beads conjugates. After incubation for overnight at 4°C on a rotating platform, the samples were centrifuged at 12,000 rpm for 1 min at 4°C and supernatants were removed. The beads were washed four times in cold PBS, boiled in 2× Laemmli Sample Buffer (125 mM Tris, pH 6.8, 4% SDS, 0.02% bromophenol blue and 20% glycerol) before being resolved by SDS–PAGE, as outlined below.

WESTERN BLOT

Cell extracts were resolved on 7.5, 10, or 15% SDS–PAGE and transferred onto nitrocellulose membranes. Membranes were incubated at room temperature for 1 h in a Tris-buffered saline with 0.1% Tween 20 (TBS-T) containing 5% skimmed milk. After being washed three times for 5 min each with TBS-T, the membrane was incubated with primary antibodies in the TBS-T containing 3% bovine serum albumin overnight at 4°C. Bound antibody was detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence using the ECL Detection Kit (Pierce).

IMMUNOFLUORESCENCE MICROSCOPY

3T3-L1 cells were grown and differentiated on glass coverslips. Cells were fixed on coverslips in 4% (wt/vol) paraformaldehyde in PBS for 15 min, followed by a 3-min permeabilization in 0.1% (vol/vol) Triton X-100 in PBS at room temperature. After blocking with PBS containing 10% normal goat serum, cells were incubated with antibodies directed against vimentin (Abcam Ltd.), phospho-PPARγ (pPPARγ; Chemicon Ltd.), and PPARγ (Santa Cruz Biotechnology, Inc.) for 1 h at 37°C. Preparations were then incubated with a

combination of tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma) as secondary antibody. Nuclei were visualized upon a 15-min incubation with 0.1 mg/ml 4,6-diamidino-2-phenylindole (DAPI). endoplasmic reticulum (ER) and mitochondria were stained with ER-Tracker™ Blue-White DPX (Molecular Probes) and Mito-Tracker® Red CMXRos (Invitrogen). For golgi staining, permeabilized cells were further incubated with a rabbit polyclonal anti-giantin antibody (Abcam) and TRITC-conjugated goat anti-rabbit IgG as secondary antibody. Cells were observed, and images were acquired with an LSM510 confocal laser-scanning microscope using a Zeiss 63X oil immersion lens.

PEPTIDE IDENTIFICATION BY MASS SPECTROMETRY AND BIOINFORMATICS ANALYSIS

The gel pieces containing polypeptides of interest were first reduced and pyridylethylated as previously described [Tsay et al., 2000]. Up to 0.2 μg of trypsin (Promega) was added to the dried gel to incubate overnight. The supernatant was removed and the gel was extracted with the adequate amount of 0.1% formic acid. After formic acid extraction, supernatant, and extracts were combined together and dried in Speed-Vac. Electrospray mass spectrometry was performed using a Finnigan Met LCQ ion trap mass spectrometer interfaced with an ABI 140D HPLC (Perkin–Elemer). A 150 × 0.5 mm PE Brownlee C18 column (Perkin-Elemer) (5 mm particle diameter, 300 pore size) with mobile phases of A (0.1% formic acid in water) and B (0.085% formic acid in aceteonitrile) were used. The peptides were then eluted using the aceteonitrile gradient and analyzed by "triple-play" experiment as described [Tsay et al., 2000]. Data interpretation and correlation between the spectra and amino acid sequences within a EST database was done by Finnigan Corporation software package, the SEQUEST Browser.

PLASMIDS

The following plasmids were kindly provided from Karsten Kristiansen and Irena Lankova: pGEX-5X-2 GST-PPARγ2 full-length, GST-PPARγ2 DEF, GST-PPARγ2 A/B, GST-PPARγ2 A/B S112A. We further subcloned the full length and truncated forms of PPARγ2 to pET-32 and pCMV-tag 2 vectors. The pET-32 plasmids were subsequently transformed in the Rosetta gami B (DE3) competent cells and protein expression were induced by 1 mM IPTG for 4 h. His-tagged PPARγ2 constructs were then purified by nickel beads for in vitro binding assay. The pCMV-tag 2 plasmids were also transfected and expressed in 293T cells. The cells were then harvested and lysates were collected for flag in vivo binding assay.

PPARγ STABILITY IN VIVO

Experiments using 3T3-L1 adipocytes were carried out in the presence of cycloheximide (5 μM) to examine the effect of vimentin knock down on the half-life of PPARγ proteins. The adipocytes were incubated with proteasome inhibitor MG132 (10 μM) in experiments designed to assay proteasome targeting of PPARγ. In these experiments, cells were preincubated with MG132 for 15–30 min prior to adding the ligand or cycloheximide.

RESULTS

IDENTIFICATION OF VIMENTIN AS A CYTOPLASMIC INTERACTING PROTEIN OF PPARγ

It has been reported that treating 3T3-L1 preadipocytes with insulin (I) and dexamethasone (Dex, D) is unable to induce adipocyte differentiation unless methylisobutylxanthine (Mix, X) is added together to stimulate the generation of endogenous PPARγ ligands via cAMP signaling pathways. This DI system allowed us to evaluate the effect of supplementation of potential PPARγ ligands in the absence of Mix. As PPARγ2 is expressed abundantly in adipocytes, we firstly treated 3T3-L1 cells with 15-keto-PGE2, a newly identified PPARγ ligand [Chou et al., 2007], and the canonical PPARγ ligand BRL to investigate protein interactions with PPARγ during adipogenesis. As shown in Figure 1A, there was a major band with molecular weight 57-kDa that showed interaction with PPARγ under both 15-keto-PGE2 and BRL treatment. To characterize the nature of this interacting protein, we isolated two protein bands (numbered 1 and 2, Fig. 1A), for liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis. The results showed that these protein bands contained several cytoskeleton proteins, with the IF vimentin being the major one (Table I). To confirm interaction with the identified protein, we performed reciprocal immunoprecipitation assays with antibodies indicated (Fig. 1B) and documented the interaction between PPARγ2 and vimentin during adipocyte differentiation in the presence of PPARγ ligands.

Since PPARγ has been shown to be phosphorylated and exported from nucleus to cytoplasm upon mitogen or ligand treatment [Burgermeister et al., 2007], we examined this interaction in lysates from different subcellular fractions. We found that the interaction of pPPARγ and vimentin occurred mainly in the membranous and cytoskeletal protein fractions (Fig. 1C). With immunocytochemistry studies, we confirmed this and found that pPPARγ and vimentin showed colocalization with endoplasmic reticulum, mitochondria and golgi apparatus (Fig. S1A–C). Thus, we verified the novel finding that vimentin is a cytoplasmic interacting protein of PPARγ.

MAPPING OF INTERACTION DOMAINS OF PPARγ WITH VIMENTIN

To gain further mechanistic insight into the binding interaction between PPARγ and vimentin, we carried out in vitro binding experiments to determine which protein domain of PPARγ is responsible for its binding to vimentin (Fig. 2A). With *Escherichia coli* expressed His-tagged proteins pull-down experiments, we found that both the AB and DEF domains of PPARγ are able to interact with vimentin (Fig. 2B), with the AB domain having a higher binding capacity (Fig. 2B).

In order to verify that this interaction occurs in other mammalian cell lines, we constructed mammalian expression plasmids containing FLAG-tagged-AB and -DEF domains of PPARγ. In vivo binding experiments in 293T cells transfected with the respective plasmids showed similar results to those seen in the in vitro protein pull-down assay (Fig. 2C). Furthermore, disruption of the phosphorylation site of PPARγ at serine112 did not interfere with its binding to vimentin (Fig. S2).

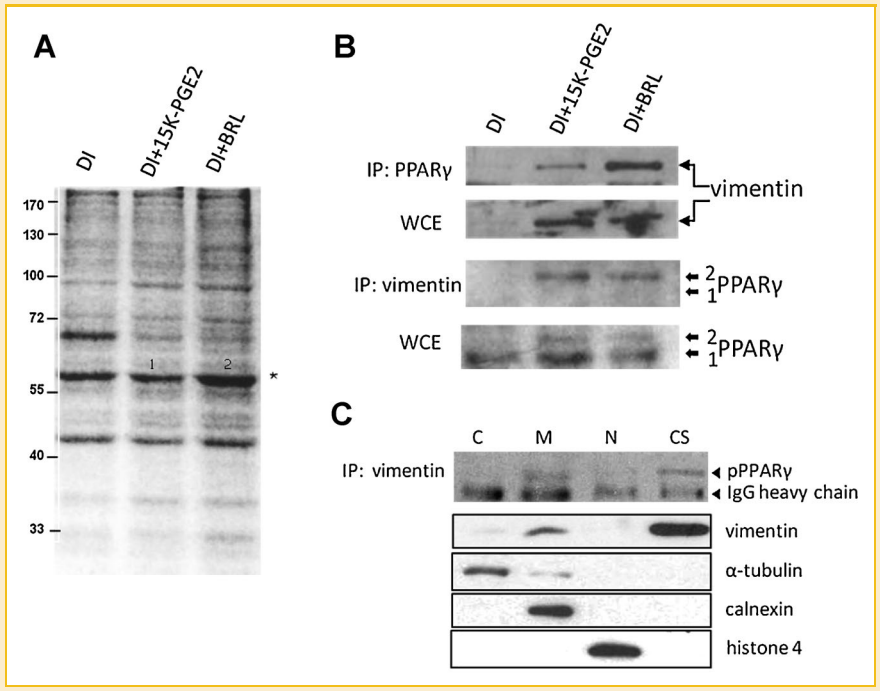

Fig. 1. Interaction of vimentin with PPARγ in 3T3-L1 adipocytes. (A) Total cell lysates were collected from the 3T3-L1 cells under DI with15-keto PGE2 or BRL treatment at Day 6 during adipocyte differentiation. Immunoprecipitation assay was performed using anti-PPARγ antibody, followed by SDS-PAGE and the gel was subsequently stained with Coomassie blue. Distinct protein bands, indicated as number 1 and 2, were subjected to LC/MS/MS analysis. (B) Total lysates were collected under DI with 15-keto PGE2 or BRL treatment at Day 6 during differentiation. For co-immunoprecipitation assays, lysates were incubated with either anti-PPARγ or anti- vimentin antibodies, and then blotted with reciprocal antibodies respectively. Whole cell extract (WCE) was loaded as a positive control. (C) 3T3-L1 adipocytes lysates were fractionated into four subcellular fractions and were immunoprecipitated with anti-vimentin and then blotted with anti-phospho- PPARγ antibodies (upper panel). The presence of respective proteins enriched for the four subcellular fractions (C, cytosol; M, membranes; N, nucleus; CS, cytoskeleton) were blotted with antibodies against α-tubulin, calnexin, histone and vimentin, respectively.

INTERACTION OF VIMENTIN AND pPPARγ IN CYTOPLASM

To evaluate the interaction between vimentin and PPARγ in selective subcellular compartments, we performed immunocytochemistry studies utilizing pre-adipocytes and well-differentiated 3T3-L1 adipocytes. In pre-adipocytes, vimentin was localized in the cytoplasm (Fig. 3A), whereas PPARγ (Fig. 3A) and pPPARγ (Fig. 3B) were localized exclusively in the nucleus. There was no colocalization of vimentin and PPARγ/pPPARγ in the 3T3-L1 pre-adipocytes (Fig. 3A and B).

TABLE I. Results the Proteins Identified With LC/MS/MS in Anti-PPARγ Immunoprecipitates

		Score
VIME_HUMAN	Vimentin: homo sapiens (human)	658
AAA61281	HUMVIM10 NID: homo sapiens	419
AAA61282	HUMVIM3 NID: homo sapiens	296
DESM_HUMAN	Desmin: homo sapiens (human)	167
Q65ZQ1_HUMAN	Anti-colorectal carcinoma heavy chain: homo sapiens (human)	110
CAA67203	HSCYTOKE1 NID: homo sapiens	92
ABA00080	DQ065670 NID: homo sapiens	80
PL0122	Ig heavy chain V-III region (TD-Vq): human (fragment)	80
BAC01495	Immunoglobulin heavy chain VHDJ region (Fragment): homo sapiens (human)	80
BAA36314	Immunoglobulin heavy chain variable region (IgM) (fragment): homo sapiens (human)	64
K2C7_HUMAN	Keratin, type II cytoskeletal 7 (cytokeratin-7) (CK-7) (keratin-7) (K7) sarcolectin): homo sapiens (human)	61
Q9H552_HUMAN	OTTHUMP00000021786: homo sapiens (human)	61
BAC02117	Immunoglobulin heavy chain VHDJ region (fragment): homo sapiens (human)	50
AAQ05536	AF471361 NID: homo sapiens	49
Q3SY84_HUMAN	Keratin 6 irs: homo sapiens (human)	44

Following 3T3-L1 adipocyte differentiation, we found that both vimentin and PPARγ/pPPARγ were gradually increased. PPARγ was almost entirely localized in the nucleus (Fig. 3C and D) whereas pPPARγ was found in both nucleus and cytosol (Fig. 3E and F). The cytosolic pPPARγ was found to increase after ligand treatment for 1 h (Fig. 3F). Interestingly, although there was no obvious colocalization between vimentin and PPARγ in the immunocytochemistry studies (Fig. 3C and D), pPPARγ showed a clear colocalization with vimentin in the cytoplasm, especially after PPARγ ligand treatment (Fig. 3E and F), indicating that the interaction of vimentin and pPPARγ occurs in cytoplasmic compartment following PPARγ activation with the agonist.

ROLE OF VIMENTIN ON UBIQUITINATION AND DEGRADATION OF PPARγ

It has been reported that ubiquitin-proteasome-dependent degradation of PPARγ is an important regulator of cellular levels of PPARγ [Hauser et al., 2000]. To examine the role of vimentin in the regulation of PPARγ protein turnover, we examined the protein half-life in 3T3-L1 cells transfected with control and vimentin

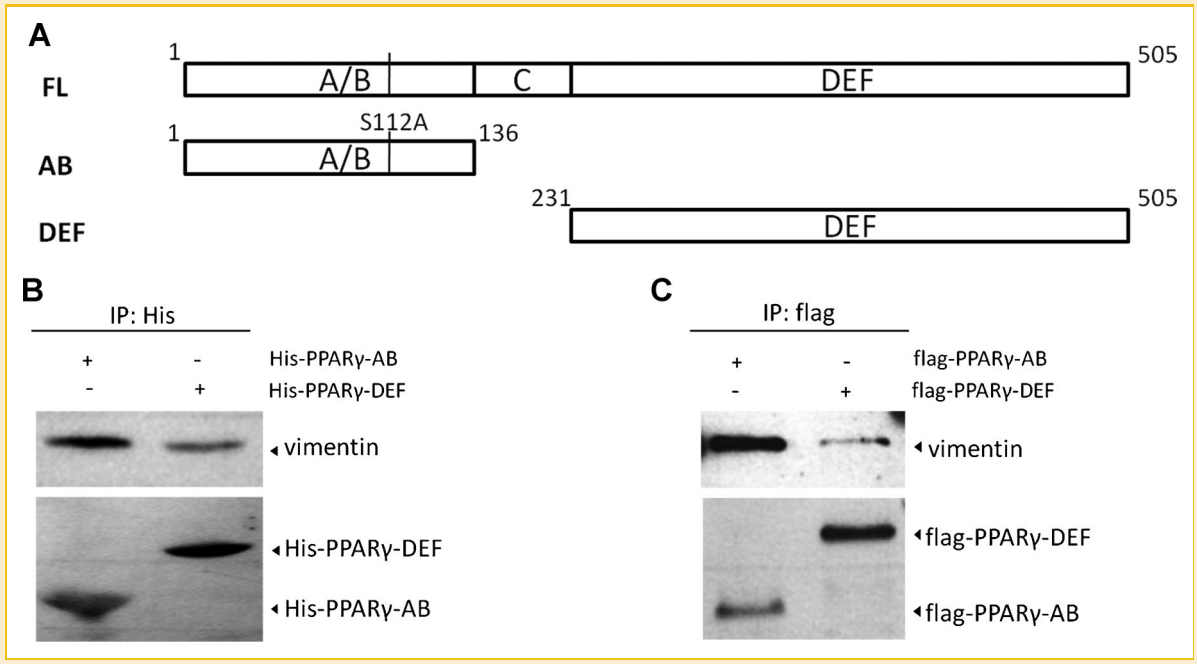

Fig. 2. Functional domains of PPARγ responsible for binding to vimentin. (A) Schematic representation of the PPARγ construct used in the subsequent experiments. (B) Different constructs encoding His-PPARγ-AB and His-PPARγ-DEF domain were transformed and expressed separately in E. coli. Immunoprecipitation was carried out with anti-his antibody followed by western blot analysis using either anti-vimentin or anti-his primary antibodies. (C) Mammalian expression plasmids encoding FLAG-PPARγ-AB and FLAG-PPARγ-DEF domain were transfected into 293T cells. Immunoprecipitation was carried out with anti-flag antibody followed by western blot analysis using either anti-vimentin or anti-flag primary antibodies.

siRNA. Although the initial PPARγ protein level was higher in vimentin knock down cells (Fig. 4A and S3), the degradation rate of PPARγ protein following ligand treatment was faster and resulted in lower protein levels in vimentin knock-down cells compared with control cells after 1 h of BRL treatment (Fig. 4A). This result was consistent with the immunocytochemistry data (Fig. 4C). The γ1 and γ2 half-lives were calculated as 1.2 and 1.35 h in control cells, but 49 and 40 min, respectively, in vimentin knock-down cells (Fig. 4B). In the presence of the proteasomal inhibitor MG132, PPARγ was maintained at a higher level. The decay experiment in Figure 4A clearly demonstrated that with the depletion of vimentin, the protein half-life of both PPARγ1 and γ2 were shortened; the lower panel showing MG132 treatment, also indicates a vimentin-mediated PPARγ proteasome-dependent degradation pathway.

Since ubiquitination is another potential mechanism underlying PPARγ proteasome degradation, we performed reciprocal immunoprecipitation experiments and analyzed proteins via immunoblotting using either anti-PPARγ or anti-ubiquitin antibody. Upon vimentin knockdown, the level of ubiquitination of PPARγ was upregulated (Fig. 5). These data indicate that vimentin may play an important role in regulating the PPARγ protein degradation rate after ligand treatment.

DISCUSSION

As a primary regulatory factor in adipocyte differentiation and expansion, PPARγ is a central component in the development of obesity. PPARγ resides primarily in the nucleus. One model proposed by Burgermeister et al. has revealed that PPARγ can be phosphorylated and exported from the nucleus upon mitogen or ligand stimulation. Here, we have demonstrated for the first time that cytosolic PPARγ directly binds to cytoskeletal vimentin after its translocation from nucleus to cytosol. Both the AB and DEF domain of PPARγ are capable of binding to vimentin, and phosphorylation of PPARγ at serine 112 is not required for this binding.

An intriguing question is now raised: what is the ultimate fate of cytosolic PPARγ after export from the nucleus and subsequent interaction with vimentin? Available knowledge, permits several possibilities. Subcellular compartmentalization is considered a major mechanism in regulating cellular signaling. The confinement of PPARγ to different subcellular compartments may serve as a temporal and spatial control of the subcellular distribution of PPARγ that separates its nuclear from cytosolic responses, which would reduce PPARγ transactivation of nuclear target genes and inhibits it genomic functions, and may also facilitate certain non-genomic effects. After transport from nucleus to cytosol, PPARγ may tether to other cell components such as G-protein coupled receptors, caveolae, lipid droplets, cytoskeleton, and, as we demonstrated here, vimentin, and may exert certain non-genomic effects. However, the potential non-genomic effects underlying the regulation of vimentin-PPARγ complexes remain to be further explored.

Vimentin has been found to function as a potential regulator of transcription, as it is able to interact with and sequester transcriptional determinants such as p53 and menin [Lopez-Egido et al., 2002; Ivaska et al., 2007]. In the human model for p53

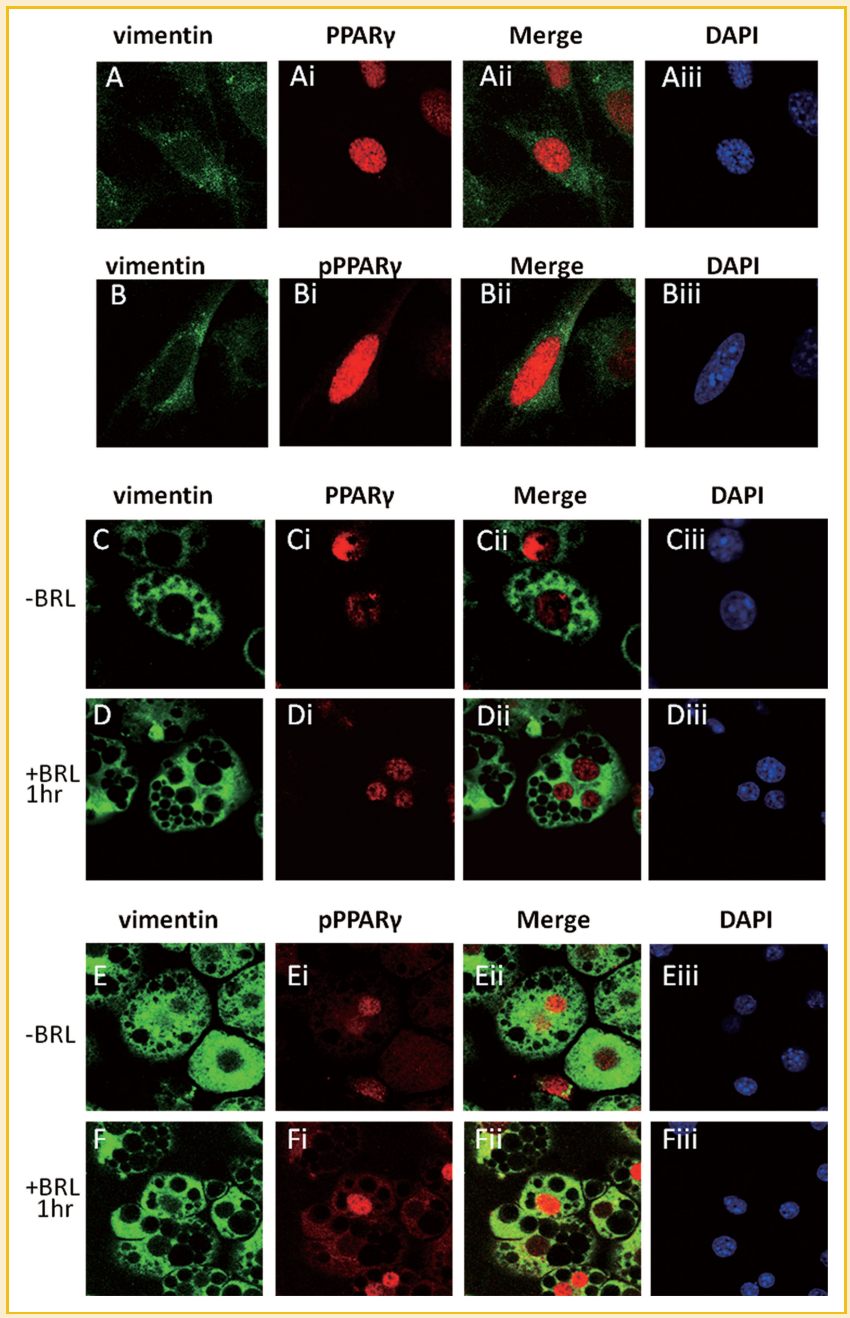

Fig. 3. Vimentin colocalized with cytosolic pPPARγ in 3T3-L1 adipocytes. (A-B) Pre-adipocytes were grown on coverslips at Day 0 (confluence) during differentiation and incubated with antibodies against vimentin, PPARγ, and pPPARγ, respectively. (C-F) Well-differentiated 3T3-L1 adipocytes (day 7) treated with BRL or not for indicated times were incubated with antibodies against vimentin, PPARγ, and pPPARγ, respectively. Nuclei were visualized by DAPI staining (blue). Images were acquired with an LSM510 confocal laser-scanning microscope using a Zeiss 63X oil immersion lens. Five independent experiments were performed, and five or more fields per sample were analyzed in each experiment and over 80% of the cells had similar staining patterns.

interaction, caspase-4 cleaves vimentin and then releases cytosolic p53 from a vimentin-p53 complex for nuclear translocation [Yang et al., 2005]. Vimentin may merely sequester cytosolic PPARγ and prevent its translocation back to the nucleus, or it may target PPARγ to other organelles, or to proteasomal degradation under certain cellular circumstances.

Intermediate filaments regulate IF-associated proteins and IF-mediated cell signaling, but they have also been shown to have a role in positioning organelles and contributing to their shape and, most importantly, their function [Toivola et al., 2005]. Vimentin has been shown to interact directly with several organelle-, membrane-, and cytoskeleton-associated proteins, to be a central participant in ER–Golgi vesicle transport and especially seems to be tightly associated with mitochondria and nuclei [Gao and Sztul, 2001; Tolstonog et al., 2001; Suzuki et al., 2002]. As we have demonstrated, the interaction of PPARγ and vimentin was observed

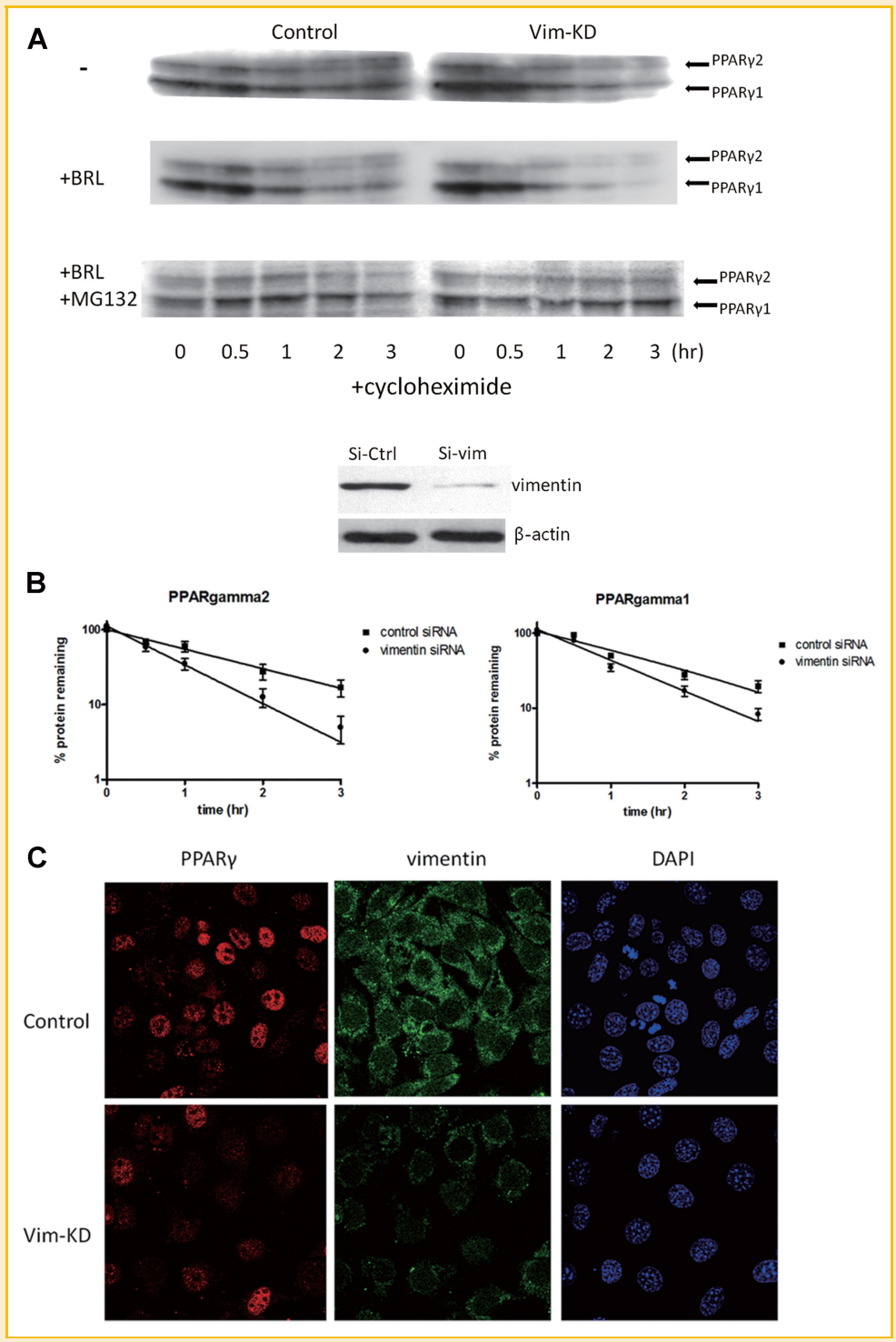

Fig. 4. The half-life of PPARγ is shortened upon vimentin knockdown. (A) Whole cell extracts were prepared from fully differentiated 3T3-L1 adipocytes after incubation in the presence of 5 μM cycloheximide for the indicated time points. Incubations were carried out in the absence or presence of BRL and MG132. This was an independent experiment performed independently for three times. The lower panel indicates the degree of vimentin knock-down. (B) The quantitative data of the degradation rate of PPARγ1 and PPARγ2 at indicated time points. (C) Early differentiated 3T3-L1 adipocytes (day 3) induced with DIM were pretreated with BRL for 1 h then fixed and incubated with antibodies against vimentin and PPARγ. Nuclei were visualized by DAPI staining (blue). Three independent experiments were performed, and five or more fields per sample were analyzed in each experiment and over 90% of the cells had similar staining patterns.

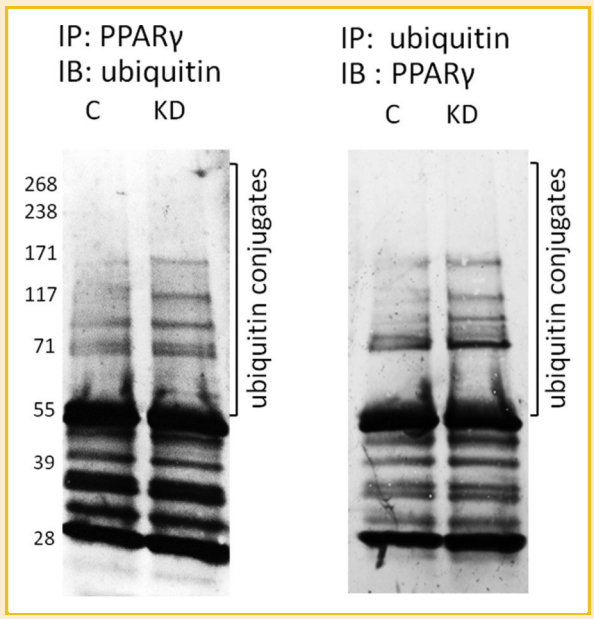

Fig. 5. Level of ubiquitinated PPARγ is elevated in vimentin knock-down 3T3-L1 cells. Whole cell extracts were harvested at the end of 1 h of BRL treatment and 1 mg of each extract was immunoprecipitated using anti-PPARγ (left) or anti-ubiquitin (right) primary antibodies. Western analysis was performed using either anti-ubiquitin (left) or anti-PPARγ (right).

at golgi, mitochondria, and ER using immunocytochemistry studies. These data imply that PPARγ may also play a certain role or exert non-genomic effects in IF-oriented modulation of organelle functions or protein targeting as a result of its association with vimentin, which requires further investigation.

Previous studies have indicated that PPARγ degradation through the ubiquitin-proteasome pathway, which is responsible for the highly regulated and energy-dependent degradation of most intracellular proteins, is an important contributor to its cellular protein levels [Hauser et al., 2000; Genini and Catapano, 2006]. Our Western blot data, which showed that PPARγ proteins were significantly accumulated under treatment of MG132, supported this finding (Fig. 4A). Protein ubiquitination–deubiquitination is a highly dynamic process and these cycles can serve to rapidly modulate protein level and function [Baek, 2003]. Moreover, the Ubiquitin-Proteasome System (UPS) regulates the abundance, activity, and subcellular localization of many transcription factors [Conaway et al., 2002; Muratani and Tansey, 2003]. Transcription factors are ubiquitinated and degraded by the proteasome and, interestingly, the process is often essential for their transactivating ability. Therefore, ubiquitin and proteasomal components were considered to play important roles in transcription [Genini et al., 2008]. We also found that in vimentin knock-down 3T3-L1 cells, PPARγ was prone to be ubiquitinated and targeted to proteasomal degradation more rapidly. The vimentin-related effect on the PPARγ turnover rate that we observed was probably due to effects on sequestration and lysosomes [Tolstonog et al., 2001], and interaction with proteasomes [Palmer et al., 1994; Arcangeletti et al., 2000]. Although the mechanisms behind the altered protein turnover rate were not well studied, our data were sufficient to prove that upon the depletion of vimentin, the activity of the PPARγ ubiquitin-proteasome degradation pathway was elevated and the half-life of PPARγ was shortened.

Vimentin was initially regarded as an exclusively intracellular cytoplasmic protein. In previous studies, vimentin has been shown to interact with several different proteins, including UPP1 [Russell et al., 2001], MYST2 [Rual et al., 2005; Stelzl et al., 2005], desmoplakin [Meng et al., 1997], plectin [Herrmann and Wiche, 1987], SPTAN1 [Brown et al., 2001], MEN1 [Lopez-Egido et al., 2002], protein kinase N1 [Matsuzawa et al., 1997], and YWHAZ [Tzivion et al., 2000]. Our current study provides the novel finding that vimentin is an interacting protein of PPARγ. However, the exact biological consequence of this interaction of vimentin and PPARγ in adipocytes remains to be further explored.

In summary, the present study has shown for the first time that upon export from the nucleus, pPPARγ interacted with the cytoskeleton protein vimentin. This is the first evidence linking the nuclear-cytoplasmic shuttling of PPARγ to a specific interacting partner in the cytosol. The depletion of vimentin from 3T3-L1 cells also shortened the half-life of PPARγ protein through more rapid ubiquitination and proteasomal degradation.

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