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Caveolin-1 Facilitates Cyclooxygenase-2 Protein Degradation

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ABSTRACT

Cyclooxygenase-2 (COX-2) plays major roles in diverse physiological and pathological processes such as inflammation and tumorigenesis. Transcriptional control of COX-2 has been extensively investigated and characterized, but its post-translational control is less clear. Here, we report a novel mechanism by which COX-2 is degraded. Protein levels of caveolin-1 (Cav-1) and COX-2 showed an inverse relation in colon cancer cell lines. COX-2 proteins in lung and colon tissues were higher in Cav-1 null mice than in wild-type mice. RNAi knockdown of Cav-1 increased COX-2 protein level and decreased ubiquitinated COX-2 accumulation. In addition, deletion of the carboxy (C)-terminus of COX-2, which contains a unique 19-amino acid segment compared with COX-1, resulted in reduced Cav-1 binding and attenuated COX-2 degradation. COX-1 and green fluorescence protein containing the C-terminus of COX-2 resulted in enhanced degradation. Our findings suggest that Cav-1 binds COX-2 in endoplasmic reticulum (ER) and carries it for degradation via ER associated degradation. The C-terminal region of COX-2 is required for Cav-1 binding and degradation. These results indicate a novel function of Cav-1 in controlling COX-2 expression, which may regulate physiological functions and have tumor suppression effects. J. Cell. Biochem. 109: 356–362, 2010.

KEY WORDS: CYCLOOXYGENASE-2; CAVEOLIN-1; PROTEIN DEGRADATION

Cyclooxygenase (COX, also known as prostaglandin H synthase) is a bifunctional enzyme that possesses bisoxygenase activity to convert arachidonic acid to prostaglandin (PG) G_2 and peroxidase activity to convert PGG_2 to PGH_2. PGH_2 is further converted to the PGs, thromboxane and prostacyclin by specific isomerases [Smith, 2008]. Two COX isoforms have been characterized. COX-1 is constitutively expressed in most mammalian cells and is considered to play "housekeeping" physiological functions. COX-2 is generally undetectable or detectable at trace levels in most mammalian cells but is highly inducible in inflammatory cells [Wu, 2007]. Furthermore, it is constitutively overexpressed in certain cancer cells [Eberhart et al., 1994; Sano et al., 1995]. COX-2 mediates inflammation and tissue damage and contributes to tumor growth and metastasis [Dubois et al., 1998]. Laboratory and clinical data from COX-2 inhibition experiments reveal that COX-2 is also essential for cardiovascular and renal functions [Adderley and Fitzgerald, 1999; Hao et al., 1999; Mukherjee et al., 2001]. Taken together, the reported findings indicate that COX-2 plays complex physiological and pathological roles and its cellular levels are tightly controlled to maintain homeostasis.

Caveolin-1 (Cav-1), a 21- to 24-kDa membrane protein, is the major protein of caveolae and is essential for caveolae structure in many cell types. Numerous studies suggest that Cav-1 plays

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important roles in many diseases such as tumorigenesis and maintenance of cardiovascular homeostasis [Schwencke et al., 2006]. Cav-1 mediates protein transportation from the endoplasmic reticulum (ER) to the cellular membrane and regulates enzymatic activity by binding and interacting with many proteins [Liu et al., 2002; Parton and Simons, 2007]. Previously, we showed that interleukin-1β (IL-1β)-induced COX-2 is colocalized and interacts with Cav-1 in fibroblasts [Liou et al., 2001]; however, the role of Cav-1 in COX-2 function remains unexplored.

Cellular COX-2 proteins are regulated at the transcriptional and posttranslational steps. Transcriptional regulation has been extensively characterized [Wu, 2005]. Less is known about regulation of COX-2 protein expression at the posttranslational level. COX-2 is associated with the luminal surface of the ER and the contiguous nuclear envelope (NE). Previous studies indicated that COX-2 is ubiquitinated and degraded in the proteasome in cytoplasm [Rockwell et al., 2000; Neuss et al., 2007]. *N*-glycosylation of one of the sites at Asn-594 is involved in ER-associated degradation (ERAD) of COX-2 [Mbonye et al., 2006]. However, the molecular mechanism by which COX-2 is degraded via the ERAD pathway is largely unknown. In this study, we delineated the involvement of Cav-1 in COX-2 degradation.

MATERIALS AND METHODS

CELL CULTURE

HT-29, HeLa and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Hyclone). H1299 cells were maintained in RPMI containing 5% FBS. Two stably transfected Cav-1-overexpressed cell lines, designated C5 and C16, and a mock-transfected cell line, designated M, were established from HT-29 cells, which constitutively express a low level of Cav-1.

ANTIBODIES AND REAGENTS

Antibodies against Cav-1, green fluorescence protein (GFP), Myc and COX-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse COX-2 monoclonal antibody was from Cayman Chemical (Ann Arbor, MI). IL-1β, ALLN, MG132, E-64d and lactacystin were from Calbiochem (La Jolla, CA). HA and Flag antibodies were from Sigma (St. Louis, MO). Human Cav-1 siRNA was from Applied Biosystems (Foster City, CA).

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS OF PGE_2 PRODUCTION

Cells were incubated in serum-free medium containing 10 μM of ^{14}C-labeled arachidonic acid at 37°C for 10 min. The media were collected, and eicosanoids were extracted by Sep-Pak Cartridge (Waters Associates, USA) and analyzed by reverse-phase HPLC, as previously described [Shyue et al., 2001].

EXPRESSION VECTOR CONSTRUCTION

COX-1 [Shyue et al., 2001] and COX-2 cDNAs (a gift of Dr. Min-Liang Kuo, National Taiwan University) [Su et al., 2004] were amplified by PCR and subcloned into pcDNA3.1/Myc-His (Invitrogen) to generate pCOX-1-Myc and pCOX-2-Myc, respectively. The COX-2 C-terminal region from 573 to 604 residues (32-aa) was amplified by PCR and subcloned into the C terminus of COX-1 in pCOX-1 and GFP in pEGFP-C3 (Clontech) to generate $pCOX\text{-}1^{+32aa}$ and $pGFP^{+32aa}$, respectively. The $pCOX\text{-}2^{\Delta 32aa}$ (deletion of the 32-aa sequence) was constructed by *Sac*I and *Bam*H1 digestion of pCOX-2, then blunt-ended, and self-ligated. Plasmids containing human Cav-1 short hairpin RNA (shRNA), pLKO-Cav-1 shRNA (pCav-1i: CCACCTTCACTGTGACGAAAT), and luciferase shRNA, a negative control (pNCi: GTACGCGGAATACTTCGA), were obtained from the National RNAi Core Facility, Academia Sinica, Taiwan. H1299 cells transfected with pCav-1i or pNCi were selected with puromycin. COX-2 cDNA was cloned into pCMV14-3-Flag, amplified by PCR, and further cloned into pXJN-HA (HA removed) (provided by Dr. S.Y. Shieh at Academia Sinica) [Ou et al., 2007] to generate pCOX-2-Flag. Cav-1 was cloned into pXJN-HA to generate pHA-Cav-1.

ANIMAL STUDIES

C57BL/6 mice (from the National Laboratory Animal Center, National Science Council, Taiwan) and Cav-1 knockout mice ($Cav1^{tm1Mls/J}$, $Cav\text{-}1^{-/-}$; The Jackson Laboratory) were housed and bred under specific pathogen-free conditions. Animal experiments were approved by the Academia Sinica Institutional Animal Care and Utilization Committee. Mice (2 male and 1 female at 8 weeks for each group) were injected intraperitoneally with PBS (control) or 2×10^7 *E. coli* BL21 (DE3) and then sacrificed at 12 or 24 h post-injection. Entire colon and lung tissues were removed and homogenized in the RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM NaF, 2 mM NaVO4, 1 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail). Following centrifugation, the extracted proteins were analyzed by Western blot analysis.

RT-PCR

RNA was isolated with TRI reagent (Sigma). 10 μg RNA was used for cDNA synthesis with oligo-dT_{21} and SuperScript III reverse transcriptase (Invitrogen). cDNAs were used for PCR amplification with the human-specific COX-2 primers 5′-CTGGCGCTCAGCCATACAGC-3′ and 5′-GGCCCTCGCTTATGATCTGTC-3′. PCR of β-actin was used as a reference with the primers 5′-CGACAACGGCTCCGGCATGTG-3′ and 5′-CGGAACCGCTCATTGCCAATGG-3′. The PCR amplification cycles were 5 cycles of 94°C, 30 s; 61°C, 30 s; 72°C, 30 s, then 25 cycles of 94°C, 30 s; 58°C, 30 s; 72°C, 30 s.

COX-2 PROMOTER ACTIVITY ASSAY

Cells were co-transfected with pXC918, a luciferase reporter vector bearing the promoter region from −918 to +49 bp of COX-2 gene (gift of Dr. W.C. Chang, National Cheng Kung University, Taiwan) [Chen et al., 2004] and the renilla luciferase-encoding plasmid pRL-tk for 48 h. Luciferase activity of cell lysates was measured by luciferase assay reagent (Promega). Relative promoter activity was measured by the ratio of luciferase to renilla activity.

IN VIVO UBIQUITINATION ASSAY

HEK293 cells were transfected with pCOX-2 and His-tagged ubiquitin construct (pHis-Ub) for 24 h and then treated with MG-132 (20 μM) for another 6 h. The cells were sonicated in buffer A (6 M guanidine–HCA, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM

imidazole, pH 8.0). After centrifugation, cell lysates were incubated with 20 μl of 50% Ni-beads (Invitrogen) at room temperature for 1 h. The precipitated beads were washed once in buffer A, once in buffer A mixed with TI buffer at 1:3 ratio and trice in TI buffer (20 mM imidazole, 0.2% Triton-X100, 25 mM Tris–HCl, pH 6.8). Bound proteins were analyzed by Western blot analysis.

IMMUNOFLUORESCENCE ASSAY

Cells were fixed in 4% paraformaldehyde in PBS for 15 min, permeablized with methanol for 15 min at room temperature, and blocked in PBS with 10% FBS for 30 min. Cells were incubated with mouse anti-Flag and rabbit anti-GFP antibodies for 1 h, washed three times, and incubated with Rhodamine-labeled donkey anti-mouse IgG and FITC-conjugated donkey anti-rabbit IgG for 1 h. The cells were washed and analyzed by confocal microscopy.

PROTEIN FRACTIONATION AND WESTERN BLOT ANALYSIS

Cells were lysed in RIPA buffer. Fractionation of subcellular proteins followed the manufacturer's protocol (Calbiochem). Cell or pull-down lysates with the appropriate amount of proteins were resolved on SDS–polyacrylamide gels, and detected by Western blot analysis as described previously [Chang et al., 2005]. β-Actin was an internal control.

IMMUNOPRECIPITATION ASSAY

Cells were lysed in RIPA buffer and sonicated at 4°C for immunoprecipitation assay as described previously [Chang et al., 2005].

RESULTS

CAV-1 SUPPRESSES COX-2 EXPRESSION IN VITRO AND IN VIVO

To determine whether Cav-1 is involved in COX-2 expression, we analyzed COX-2 protein level in HT-29 colon cancer cells with and without stable transfection of Cav-1. Cav-1 proteins were expressed at low levels in untransfected or mock-transfected HT-29 cells. Two stably transfected cell lines, C5 and C16, expressed a high level of Cav-1 (Fig. 1A). Cultured in the absence of FBS, COX-2 protein level was low in control cells and barely detectable in C5 or C16 cells (Fig. 1A); the level was increased in control cells but remained barely detectable in C5 or C16 cells treated with FBS or IL-1β (Fig. 1A). Time-course experiments revealed that COX-2 was barely detectable throughout the 24-h period of IL-1β treatment in C5 cells (Fig. 1B). By contrast, COX-2 protein level was increased at 6 h and persisted for 24 h following IL-1β treatment in control cells. PGE_2, a major COX-2 metabolite in HT-29 cells, was detectable in mock but not in C5 or C16 cells (Fig. 1C). These results reveal an inverse relation between Cav-1 and COX-2 protein levels in colon cancer cell lines. To gain insight into the role of Cav-1 in controlling COX-2 protein expression, we evaluated the effect of a selective Cav-1 siRNA on COX-2 protein level in H1299 cells, which constitutively express a high level of Cav-1 (Fig. 1D). Basal COX-2 was undetectable in this cell line (data not shown). H1299 cells were transfected with Cav-1 shRNA (pCav-1i) for 48 h, then transfected with pCOX-2-Myc. Cav-1 protein level was suppressed >70% with shRNA, whereas control shRNA had no effect on Cav-1 level (Fig. 1D). Cav-1 shRNA

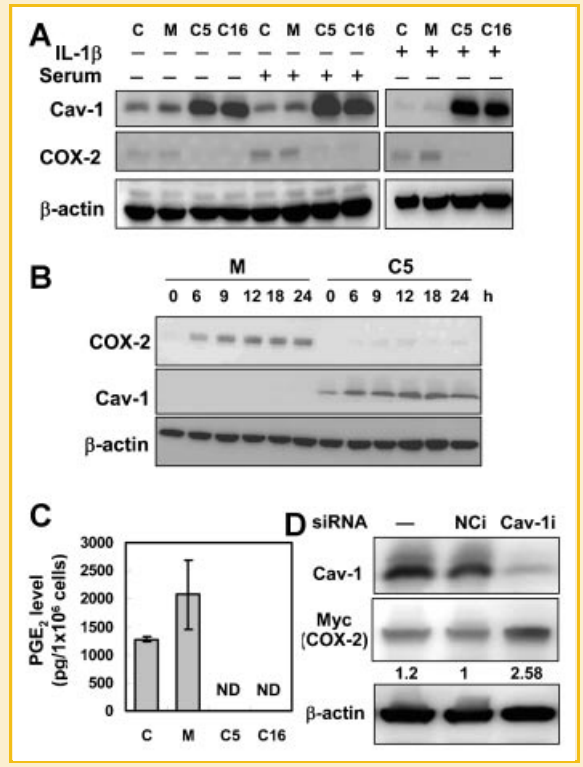

Fig. 1. Down-regulation of COX-2 by Cav-1 in colon cancer cells. A: Western blot analysis of Cav-1 and COX-2 in untransfected (C), mock-transfected (M) and Cav-1 stably transfected HT-29 cell lines (C5 and C16) cultured in serum-free media and treated with 10% FBS or IL-1β (2 ng/ml) for 24 h. β-actin as a loading control. B: Western blot analysis of COX-2 expression in cells cultured in serum-free medium and stimulated with IL-1β for 0–24 h. C: Cells were incubated with C^{14}-arachidonic acid for 10 min, and PGE_2 in the medium was analyzed by reverse-phase HPLC. Each bar denotes mean ± SEM (n = 3). ND, not detected. D: Western blot analysis of H1299 cells transfected with pCav-1i or control RNAi (NCi) for 48 h, then co-transfected with Myc-tagged COX-2 and GFP (as a transfection reference). Relative COX-2 levels were measured by densitometry and indicated as ratios normalized to NCi.

increased the COX-2-Myc protein level by ~2.5-fold over the control in H1299 cells (Fig. 1D).

To confirm that Cav-1 controls COX-2 expression in vivo, we analyzed COX-2 protein level in wild-type (WT) and Cav-$1^{-/-}$ mice. In lung tissue, basal COX-2 protein level was higher in Cav-$1^{-/-}$ than in the WT (Fig. 2A). Intraperitoneal injection of *E. coli* increased COX-2 protein in WT and Cav-$1^{-/-}$ lung tissue in a time-dependent manner (Fig. 2A). Similarly, in colon tissue, the basal COX-2 protein level was several-fold higher in Cav-$1^{-/-}$ than in the WT (Fig. 2B). *E. coli* challenge for 24 h increased COX-2 levels in both WT and Cav-$1^{-/-}$ colon tissue (Fig. 2B).

COX-2 DEGRADATION VIA THE PROTEASOME PATHWAY

To determine whether Cav-1 controls COX-2 expression at the transcriptional level in HT-29 cells, we measured COX-2 mRNA by RT-PCR and analyzed COX-2 promoter activity using a COX-2 promoter-luciferase construct. C5 and C16 cells did not differ from control cells in COX-2 mRNA and promoter activity (Fig. 3A,B), which implies that Cav-1 alters COX-2 protein stability. Proteasome

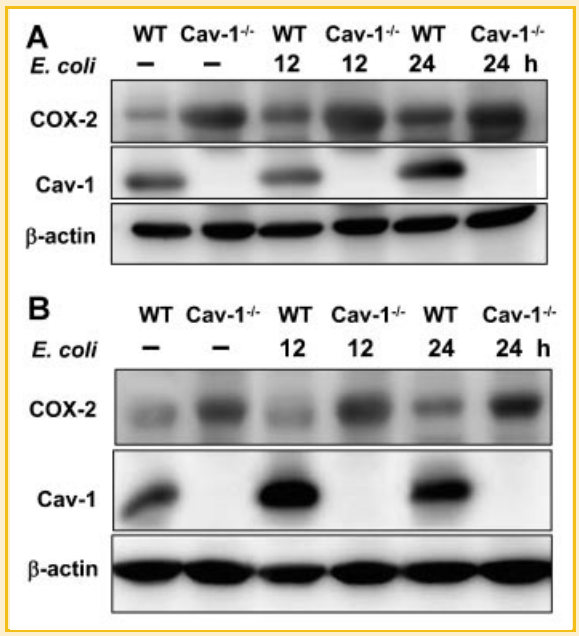

Fig. 2. Elevation of COX-2 proteins in Cav-1$^{-/-}$ mice. Wild-type (WT) and Cav-1$^{-/-}$ mice were challenged with *E. coli* for 12 or 24 h. Western blot analysis of COX-2 and Cav-1 protein in lung (A) and colon (B) tissues.

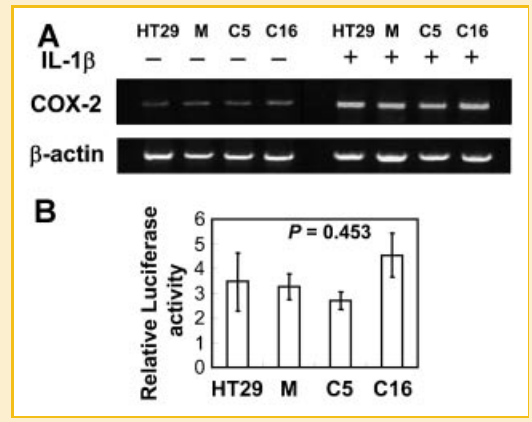

Fig. 3. Regulation of COX-2 protein expression is transactivation independent. HT-29 cells were treated with or without IL-1β for 6 h. A: COX-2 mRNA levels were analyzed by RT-PCR. B: COX-2 promoter activity was analyzed by transfection assay. Statistical analysis was performed by one-way analysis of variance. Each bar denotes mean ± SEM (n = 3).

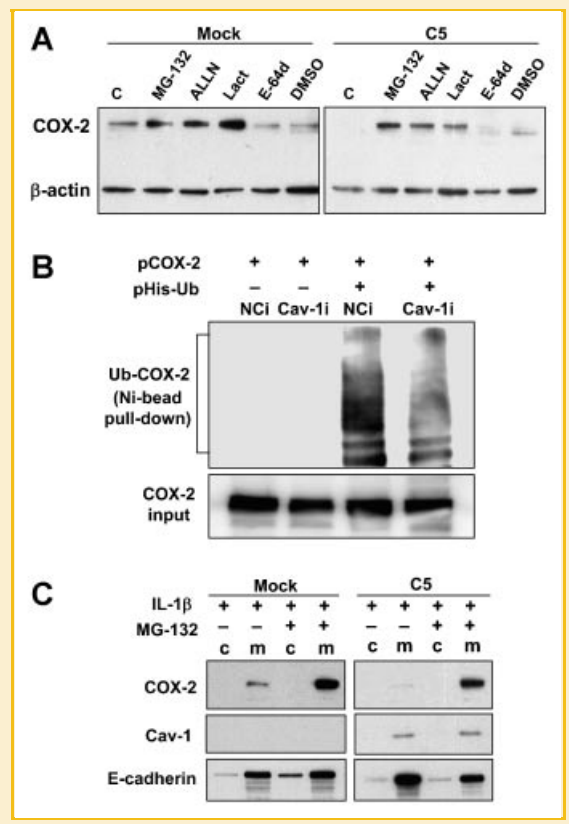

Fig. 4. COX-2 degradation via the proteasome pathway. A: Western blot analysis of cells stimulated with IL-1β for 6 h, then proteasome inhibitor MG-132 (10 μM), ALLN (20 μM), or lactacystin (Lact, 10 μM) or cysteine protease inhibitor E-64d (50 μM) was added for 9 h. B: Western blot analysis of COX-2 from HEK293 cells transfected with Cav-1 siRNA for 24 h followed by pCOX-2 and pHis-Ub for 24 h. After MG132 treatment, His-conjugated proteins were isolated using Ni-beads and analyzed with anti-COX-2 antibody. C: Co-localization of COX-2 and Cav-1 to membrane fractions. Mock and C5 cells were treated with IL-1β with and without MG-132 for 6 h. Western blot analysis of COX-2 and Cav-1 expression in cytosolic (c) and membrane fractions (m). E-cadherin was analyzed as the membrane marker.

inhibitors (MG-132, ALLN and lactacystin) increased COX-2 protein levels in mock-transfected cells, but the cysteine protease inhibitor (E-64d) had no effect (Fig. 4A). All three proteasome inhibitors increased COX-2 protein level considerably in Cav-1-overexpressed C5 cells. We next examined effect of Cav-1 on COX-2 ubiquitination. HEK293 cells were transfected with pHis-ubiquitin and pCOX-2-Flag for in vivo ubiquitination assay. As shown in Figure 4B, suppression of Cav-1 protein level by siRNA attenuated the ubiquitinated COX-2 accumulation (Fig. 4B). These results suggest that Cav-1 facilitates COX-2 protein degradation via the proteasome pathway. In addition, IL-1β-induced COX-2 was detected in the membrane fraction of mock-transfected but not C5 cells (Fig. 4C). Following MG-132 treatment, COX-2 was increased in the membrane fraction of mock-transfected and C5 cells. Cav-1 detected only in the membrane fraction of C5 cells was not influenced by MG-132 (Fig. 4C). Because COX-2 proteins are primarily localized to the ER and NE, Cav-1 may co-localize with COX-2 to these subcellular organelles. Taken together, the findings suggest that Cav-1 may be involved in COX-2 protein degradation in the ER and NE.

THE CARBOXY-TERMINUS OF COX-2 IS INVOLVED IN CAV-1-MEDIATED PROTEIN DEGRADATION

The carboxy (C)-terminal 19 amino acids (aa) of COX-2, which are absent in COX-1, harbors a glycosylation site at Asn-594, which was reported to be required for COX-2 degradation via ERAD [Mbonye et al., 2006]. To determine whether this region is involved in interaction with Cav-1, we constructed a COX-2 C-terminal 32-aa deletion mutant (COX-2$^{\Delta 32aa}$), with Asn-594 and its adjacent 18-aa deleted, and a COX-1 insertion mutant (COX-1^{+32aa}), with the 32-aa

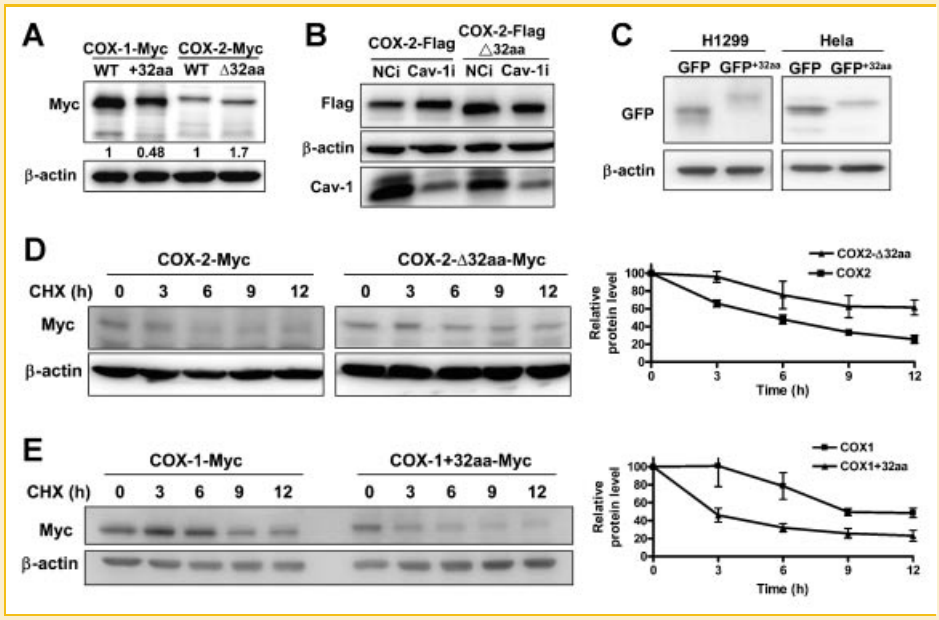

Fig. 5. Involvement of COX-2 C-terminal region in protein stability. A: Western blot analysis of H1299 cells transfected with Myc-tagged WT COX-1, COX-1^{+32aa}, WT COX-2, or COX-$2^{\Delta 32aa}$ with anti-Myc antibody. Relative COX-1 (WT vs. +32aa) and COX-2 (WT vs. Δ32aa) protein levels were measured by densitometry and indicated as ratios normalized to those of respective WT. B: H1299 cells were transfected with Cav-1 siRNA (Cav-1i) or control siRNA (NCi) for 24 h, then transfected with Flag-tagged WT COX-2, or COX-$2^{\Delta 32aa}$ for Western blot analysis. C: Western blot analysis of GFP expression in H1299 or HeLa cells transfected with GFP or GFP^{+32aa}. D and E: H1299 cells were transfected with the indicated plasmids for 24 h, then treated with cycloheximide (CHX) (20 μg/ml) for 3–12 h. Western blot analysis of time-dependent COX-2 (D) or COX-1 (E) expression with anti-Myc antibody. Relative COX-2 and COX-1 protein levels were determined by densitometry and plotted versus time (h). Data are means ± SEM (n = 3).

inserted into the C terminus of COX-1. Transfection of H1299 cells with Myc-tagged COX-$2^{\Delta 32aa}$ increased COX-2-Myc level as compared with WT COX-2, whereas transfection with Myc-tagged COX-1^{+32aa}, the level of COX-1-Myc was lower than that in WT COX-1 (Fig. 5A). We next evaluated the effect of Cav-1 siRNA on WT and COX-$2^{\Delta 32aa}$ protein expression. H1299 cells were transfected with Cav-1 siRNA for 24 h, followed by Flag-tagged WT COX-2 or COX-$2^{\Delta 32aa}$. WT COX-2 level was higher in cells transfected with Cav-1 siRNA than control cells (Fig. 5B, lane 1 vs. lane 2). Flag-tagged COX-$2^{\Delta 32aa}$ protein level was higher than Flag-tagged WT COX-2 in H1299 cells transfected with control siRNA (Fig. 5B, lane 1 vs. lane 3). Flag-tagged COX-$2^{\Delta 32aa}$ protein was not significantly altered by Cav-1 siRNA treatment (Fig. 5B, lane 3 vs. lane 4). These results suggest that deletion of the C-terminal 32-aa of COX-2 impedes Cav-1-mediated protein degradation. Insertion of the 32-aa peptide in the C terminus of GFP also reduced GFP protein level in H1299 and HeLa cells (Fig. 5C). Deletion of 32-aa from the C terminus of COX-2 prolonged the half-life of COX-2 (Fig. 5D), whereas insertion of the 32-aa into COX-1 shortened the half-life of COX-1 (Fig. 5E). These results confirm that the C-terminal 32-aa is involved in Cav-1-mediated COX-2 degradation.

To examine whether attenuation of COX-$2^{\Delta 32aa}$ degradation was due to alteration of protein localization, HEK293 cells were co-transfected with pER-GFP (ER-localized GFP with a KDEL sequence) plus pCOX-2-Flag or pCOX-$2^{\Delta 32aa}$-Flag, and their localization was detected by confocal microscopy. As shown in Figure 6, COX-2 without the 32-aa colocalized with ER-GFP in the ER, as was the WT COX-2, which suggests that the ER and NE targeting of COX-2 is not altered by the deletion of the C terminus.

We next determined whether the C-terminal 32-aa is involved in COX-2 interaction with Cav-1. HA-Cav-1 was co-expressed with COX-2-Flag or COX-$2^{\Delta 32aa}$-Flag in HEK293 or H1299 cells. The amounts of plasmid DNA of pCOX-2-Flag and pCOX-$2^{\Delta 32aa}$-Flag used for transfection were adjusted to achieve similar protein expression levels (Fig. 7). Proteins were pulled down with an anti-HA antibody, and COX-2 or Cav-1 in the immunoprecipitates was

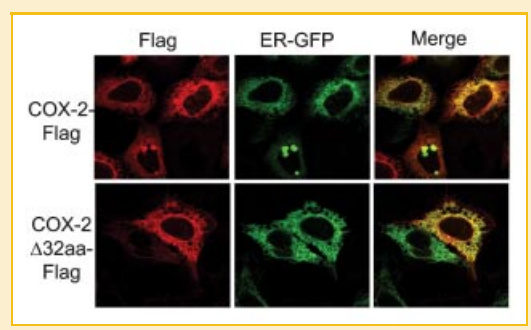

Fig. 6. ER localization of COX-2 and COX-$2^{\Delta 32aa}$. HEK293 cells expressing ER-GFP and COX-2-Flag or COX-$2^{\Delta 32aa}$-Flag were double-stained with Rhodamine (against Flag) and FITC (against GFP) and examined by confocal microscopy. Image overlay shows the colocalization of both proteins. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

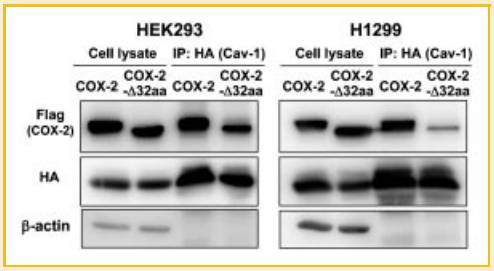

Fig. 7. Involvement of COX-2 C-terminal region in Cav-1 interaction. HEK293 and H1299 cells were co-transfected with HA-Cav-1 and Flag-tagged COX-2 or COX-2$^{\Delta 32aa}$. Cell lysates were immunoprecipitated with HA antibody and examined by Western blot analysis with Flag and HA antibodies.

detected by Western blot analysis. COX-2$^{\Delta 32aa}$ in the immunocomplex pulled down by HA antibody was markedly reduced in both HEK293 and H1299 cells (Fig. 7), which suggests that the C-terminal 32-aa of COX-2 is required for Cav-1 interaction.

DISCUSSION

Cav-1 interacts with lipids and a variety of proteins [Liu et al., 2002]. It serves as a transporter of cholesterol and functions as a key element in membrane trafficking and signal transduction [Razani and Lisanti, 2001; Schwencke et al., 2006; Parton and Simons, 2007]. Cav-1 is proposed to re-cycle from caveolae to form soluble Cav-1, which may enter the lumen of the ER or anchor to the cytosolic surface of the ER [Liu et al., 2002]. In this study, we provide evidence that Cav-1 binds an ER protein, COX-2, and facilitates its degradation. Our data reveal that Cav-1 mediates not only in vitro but also in vivo COX-2 degradation, as demonstrated by markedly increased COX-2 expression in both lung and colon tissues of Cav-1$^{-/-}$ mice and in colon cancer cells. These in vivo and in vitro data suggest that Cav-1 plays an important role in regulating the basal and induced COX-2 levels in the normal lung and colon, as well as in carcinoma cells. COX-2 is a major mediator of inflammation and tissue injury. Its expression is upregulated by bacterial infection of tissues such as lung and colon. Our results imply that Cav-1 plays an important physiological role in defending against bacteria-induced lung and colon tissue inflammation and tissue injury through degrading COX-2 thereby suppressing COX-2 derived pro-inflammatory mediators such as PGE_2.

Cav-1 was reported to be involved in degradation of inducible nitric oxide synthase (iNOS) in the cytosol, but the molecular mechanism is unclear [Felley-Bosco et al., 2000]. In contrast to the situation for COX-2, the level of iNOS was down-regulated in Cav-1$^{-/-}$ macrophages induced by *E. coli* (unpublished data), which suggests a different regulatory mechanism of iNOS by Cav-1. Recently, Cav-1 was suggested to suppress COX-2 expression through down-regulation of mRNA level [Rodriguez et al., 2009]. However, for an unknown reason, our results did not show the regulation of COX-2 mRNA expression by Cav-1 in HT-29 cells, because overexpression of Cav-1 in C5 and C16 cells did not alter COX-2 mRNA levels with or without IL-1β induction as compared with control cells (Fig. 3).

The C terminus of COX-2, which contains an Asn-594 glycosylation site, was proposed to be involved in degradation via ERAD [Mbonye et al., 2006]. Our results show that this region facilitates COX-2 degradation because its deletion prolonged the half-life of COX-2 protein. We provide novel information that this region is involved in Cav-1 binding. Deletion of this region substantially reduced COX-2 binding to Cav-1 in H1299 and HEK293 cells. As well, our results reveal that the C-terminal region of COX-2 is not required for membrane anchoring because its deletion does not alter COX-2 localization. These results support the notion that Cav-1 is a component of the ERAD machinery. We propose that Cav-1 carries the target ER protein to the ERAD channel for transport to the cytosol for ubiquitination and proteasome degradation.

To gain insight into the involvement of the COX-2 C-terminal 32-aa region in protein degradation, we evaluated the influence of this segment on protein levels by transfecting cells with insertion mutants of COX-1 and GFP. Insertion of the 32-aa segment into COX-1 and GFP reduced protein levels (Fig. 5) as compared with the WT protein level in both H1299 and Hela cells. These results imply that the COX-2 C-terminal region per se facilitates protein degradation. It is of interest to note that WT COX-1 also binds Cav-1 in HEK293 cells, which suggests that COX-1 bears the Cav-1 binding motif. However, Cav-1 binding to this putative motif does not enhance COX-1 degradation (data not shown).

The Cav-1-mediated ERAD represents a novel mechanism for ER protein degradation and is crucial for maintaining a homeostatic level of biologically active proteins such as COX-2 in cells. The biochemical process by which Cav-1 mediates COX-2 degradation is largely unknown. Our results show an interaction of Cav-1 with COX-2, which probably occurs at the ER and NE because both proteins were detected in membrane fractions of C5 cells over-expressing Cav-1 especially when proteasome degradation was blocked with pharmacological inhibitors. Previous, we have shown that COX-2 binds to ER and NE lumen through interaction of a stretch of hydrophobic residues with the lumen membrane [Liou et al., 2001]. In this study, we demonstrated that suppression of Cav-1 protein level by siRNA decreased ubiquitinated COX-2 level. These results suggest that Cav-1 may serve as a chaperon to carry COX-2 to the ERAD for subsequent ubiquitination and proteasome degradation. Since COX-2 levels was regulated in lung and colon of Cav-1$^{-/-}$ mice, there should be other protein degradation machinery in cells, which mediates COX-2 protein degradation.

Cav-1-mediated COX-2 degradation is crucial for maintaining COX-2 at the homeostatic level. A certain level of COX-2 is believed to be required for carrying out proper physiological functions. However, excess and prolonged COX-2 expression may cause uncontrolled inflammatory responses and tissue damage. Strong evidence suggests that COX-2 overexpression alters cell properties toward malignancy and promotes tumorigenesis [Dubois et al., 1998]. Recent studies suggest that COX-2 is one of the critical mediators regulating vascular remodeling in lung metastasis [Gupta et al., 2007]. Cav-1 may thus be a gatekeeper to maintain COX-2 at the physiological level in normal cells. Because Cav-1 expression is suppressed in certain cancer cells in which COX-2 is constitutively expressed, COX-2 overexpression in cancer cells may be attributed

to deficiency in the gatekeeper function of Cav-1. Although Cav-1 is increasingly considered to be a tumor suppressor gene [Bender et al., 2000; Lin et al., 2007], the mechanism by which it suppresses tumors is largely unknown. Our results suggest that its tumor suppressing effects may be facilitating degradation of pro-neoplastic and pro-inflammatory proteins such as COX-2.

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