A Cyclooxygenase-2-dependent Prostaglandin E\textsubscript{2} Biosynthetic System in the Golgi Apparatus* 

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**Background:** When cyclooxygenases-1 and -2 (COXs-1 and -2) are co-expressed, COX-2 can function, whereas COX-1 is latent. COX-2 is degraded via endoplasmic reticulum (ER)-associated degradation (ERAD) following post-translational glycosylation of Asn-594. COX-1 and COX-2 are found in abundance on the luminal surfaces of the ER and inner membrane of the nuclear envelope. Using confocal immunocytofluorescence, we detected both COX-2 and microsomal PGE synthase-1 (mPGES-1) but not COX-1 in the Golgi apparatus. Inhibition of trafficking between the ER and Golgi retarded COX-2 ERAD. COX-2 has a C-terminal STEL sequence, which is an inefficient ER retention signal. Substituting this sequence with KDEL, a robust ER retention signal leads to sluggish Golgi to ER transit of COX-2. The Asn-594-linked glycan is trimmed prior to retrograde COX-2 transport to the ER for ERAD. Having an inefficient ER retention signal, concentrated COX-2 in the ER where it was stable and slowly glycosylated on Asn-594. Native COX-2 and a recombinant COX-2 having a Golgi targeting signal but not native COX-1 exhibited efficient catalytic coupling to mPGES-1. We conclude that N-glycosylation of Asn-594 of COX-2 occurs in the ER, leading to anterograde movement of COX-2 to the Golgi where the Asn-594-linked glycan is trimmed prior to retrograde COX-2 transport to the ER for ERAD. Having an inefficient ER retention signal leads to sluggish Golgi to ER transit of COX-2. This permits significant Golgi residence time during which COX-2 can function catalytically. Cytosolic phospholipase A\textsubscript{2a}, which mobilizes arachidonic acid for PG synthesis, preferentially translocates to the Golgi in response to physiologic Ca\textsuperscript{2+} mobilization. We propose that cytosolic phospholipase A\textsubscript{2a}, COX-2, and mPGES-1 in the Golgi comprise a dedicated system for COX-2-dependent PGE\textsubscript{2} biosynthesis.

**Results:** Significant amounts of COX-2, microsomal PGE synthase-1, and cytosolic PLA\textsubscript{2a}, but not COX-1 are in the Golgi. 

**Conclusion:** Cytosolic PLA\textsubscript{2a}, COX-2, and microsomal PGE synthase-1 comprise a unique COX-2-dependent PGE\textsubscript{2} biosynthetic system in the Golgi.

**Significance:** COX-2 and COX-1 can function in different subcellular compartments.

Cyclooxygenases (COXs) catalyze the committed step in prostaglandin (PG) biosynthesis. COX-1 is constitutively expressed and stable, whereas COX-2 is inducible and short lived. COX-2 is degraded via endoplasmic reticulum (ER)-associated degradation (ERAD) following post-translational glycosylation of Asn-594. COX-1 and COX-2 are found in abundance on the luminal surfaces of the ER and inner membrane of the nuclear envelope. Using confocal immunocytofluorescence, we detected both COX-2 and microsomal PGE synthase-1 (mPGES-1) but not COX-1 in the Golgi apparatus. Inhibition of trafficking between the ER and Golgi retarded COX-2 ERAD. COX-2 has a C-terminal STEL sequence, which is an inefficient ER retention signal. Substituting this sequence with KDEL, a robust ER retention signal, concentrated COX-2 in the ER where it was stable and slowly glycosylated on Asn-594. Native COX-2 and a recombinant COX-2 having a Golgi targeting signal but not native COX-1 exhibited efficient catalytic coupling to mPGES-1. We conclude that N-glycosylation of Asn-594 of COX-2 occurs in the ER, leading to anterograde movement of COX-2 to the Golgi where the Asn-594-linked glycan is trimmed prior to retrograde COX-2 transport to the ER for ERAD. Having an inefficient ER retention signal leads to sluggish Golgi to ER transit of COX-2. This permits significant Golgi residence time during which COX-2 can function catalytically. Cytosolic phospholipase A\textsubscript{2a}, which mobilizes arachidonic acid for PG synthesis, preferentially translocates to the Golgi in response to physiologic Ca\textsuperscript{2+} mobilization. We propose that cytosolic phospholipase A\textsubscript{2a}, COX-2, and mPGES-1 in the Golgi comprise a dedicated system for COX-2-dependent PGE\textsubscript{2} biosynthesis.

Prostaglandins (PGs)\textsuperscript{2} are an important class of lipid mediators that modulate many key physiological processes including immunity (1), reproduction (2), and renal and cardiovascular homeostasis (3, 4). Two isoforms, prostaglandin-H syntheses-1 and -2, catalyze the committed step in the biosynthesis of PGs from arachidonic acid (AA). These enzymes are commonly referred to as cyclooxygenases-1 and -2 (COXs-1 and -2), and this terminology is used in the present report. The formation of PGH\textsubscript{2} from AA involves two distinct steps occurring at separate COX and peroxidase active sites of prostaglandin-H syntheses (for recent reviews see, Refs. 5–7). Briefly, after AA is mobilized from the sn2 position of membrane glycerophospholipids by one or more phospholipase A\textsubscript{2} (PLA\textsubscript{2}) species (for recent reviews, see Refs. 8–10), AA is oxygenated to form PGG\textsubscript{2} in the COX site. Newly formed PGG\textsubscript{2} then moves to the peroxidase site of the enzyme or another peroxidase where the 15-hydroperoxy group of PGG\textsubscript{2} is reduced, yielding PGH\textsubscript{2}. Subsequently, PGH\textsubscript{2} is converted by a downstream prostaglandin synthase such as microsomal PGE synthase-1 (mPGES-1) into a biologically active prostanooid as detailed in a recent review (7).

Both isoforms catalyze the conversion of AA to PGH\textsubscript{2} with similar K\textsubscript{m} and V\textsubscript{max} values, and the critical catalytic residues are the same in COX-1 and COX-2 (5–7). Although both isoforms are sequence homodimers, they each function as conformational heterodimers comprising allosteric and catalytic monomers with only one of the two COX sites catalyzing a reaction at any given time (11–16). Recent studies by our group have shown that the activities of both COX-1 and COX-2 are attenuated or enhanced, respectively, by non-substrate fatty acids such as palmitic acid that function allosterically to regulate COX activity (14, 16, 17).

COX-1 and COX-2 are N-glycosylated, ER-resident, homodimeric enzymes that exhibit about 60% sequence identity within a species. The most obvious sequence difference between COX-1 and COX-2 is the presence in COX-2 of a putative C-terminal STEL ER retention signal, which mobilizes arachidonic acid for PG synthesis, preferentially translocates to the Golgi in response to physiologic Ca\textsuperscript{2+} mobilization. We propose that cytosolic phospholipase A\textsubscript{2a}, COX-2, and mPGES-1 in the Golgi comprise a dedicated system for COX-2-dependent PGE\textsubscript{2} biosynthesis.
growth factor (EGF)-like domain, a membrane binding domain, and a catalytic domain (Fig. 1A). The function of the N-terminal EGF-like domain, part of which lies at the dimer interface, remains unclear. As first proposed by Garavito and co-workers (18), the membrane binding domains serve to anchor COXs to one face of a lipid bilayer. Both COX-1 and COX-2 have been shown to be bound to the luminal surfaces of the lipid bilayer of the ER and the contiguous inner membrane of the nuclear envelope (19–23). COX-1 appears somewhat more concentrated in the ER, and COX-2 appears more concentrated on the nuclear envelope (24). The catalytic domain is located toward the C terminus, includes about 75% of the entire sequence, and contains the key COX and peroxidase active site residues. Despite their many similarities in structure and catalytic activities, COX-1 and COX-2 display temporally distinct patterns of gene expression and mRNA and protein turnover (25). The evolutionary forces that have driven many species to have two biochemically similar COX isomers associated with different physiologic events remain an enigma (26, 27).

With respect to protein expression, COX-1 is a stable protein (28–30) that is constitutively expressed in many but not all mammalian cells (31). PGs formed via COX-1 are involved in homeostatic housekeeping events such as thrombosis, parturition, and regulation of renal water balance. In contrast, COX-2 is usually expressed transiently upon treatment of cells with growth factors or proinflammatory stimuli (25) that promote cell division or differentiation. The expression of COX-2, although undetectable in most cells, can increase and decrease in a matter of hours often in association with inflammation. Regulation of COX-2 protein concentrations occurs at multiple transcriptional, post-transcriptional, translational, and post-translational levels (25, 29, 32). COX-2 overexpression is associated with several forms of cancer (33, 34).

The present report focuses on the post-translational processing and degradation of COX-2 in the context of COX-2 trafficking and the subcellular localization and functioning of COX-2. We have reported that properly folded COX-2 can be degraded via two distinct pathways including AA turnover-dependent degradation and constitutive degradation involving the ER-associated degradation (ERAD) pathway (29, 30). Others have shown that COX-2 can be ubiquitinated and degraded through the 26 S proteasome (32, 35). ERAD involves a series of related but different events that include detecting misfolded proteins bound for degradation, translocating these proteins into the cytosol, and degrading the proteins via the 26 S proteasome (for recent reviews see, Refs. 36–40). In general, the ERAD pathway functions as a quality control process to monitor protein folding and to remove aberrant proteins from the ER. However, in the case of COX-2, the ERAD pathway serves as one mechanism to control the level of active COX-2.

Previously, we identified a 27-amino acid instability motif involving residues Glu-586 to Lys-612 of COX-2 that acts as a degradation signal or “degron” (39). This degron includes the unique 19-amino acid sequence near the C terminus of COX-2 that is essential for regulating N-glycosylation of Asn-594 and mediating COX-2 entry into the ERAD pathway. Deletion of the 19-amino acid sequence Asn-594 to Lys-612 (Fig. 1A) of human (hu) COX-2 generates a degradation-resistant form, whereas inserting this sequence into the corresponding position in COX-1 markedly destabilizes the resulting COX-1 (28). The COX-2 degron appears to control the entry of COX-2 into the ERAD pathway. The effect parallels the function that the multiple AUUUA elements in the 3′-untranslated region of COX-2 mRNA play in its rapid cleavage (41).

There are four known N-glycosylation sites in COX-2 (42) (see Fig. 1A). N-Glycosylation of Asn-594 within the degron occurs post-translationally and is central to its ERAD (29, 30, 35). An N594A mutation in COX-2 blocks enzyme degradation. During the process of distinguishing between terminally misfolded proteins and properly folded intermediates, α-mannosidases remove mannoses to trim the Asn-linked Manα-(GlcNAc)2 oligosaccharides, generating an appropriate ERAD signal (38, 43, 44). Apparently, this occurs with the N-glycosylation moiety linked to Asn-594 of COX-2. Importantly, N-glycosylation of Asn-594 is necessary but not sufficient for proteasomal degradation of COX-2; additional segments of the degron are involved (28, 29). Accordingly, we searched for other factors involved in the ERAD of COX-2. During the course of these studies, we discovered that replacing the STEL sequence at the C terminus of COX-2 with KDEL, a more typical and robust ER retention signal (45), yielded a degradation-resistant COX-2. This led us to explore the role of protein trafficking in the degradation of huCOX-2. Data reported here indicate that a significant amount of huCOX-2 resides in the Golgi apparatus, whereas little or no COX-1 is detectable in the Golgi. Additionally, we found that one of the enzymes downstream of COX-2 in PG biosynthesis, mPGES-1, is also present in the Golgi and that COX-2 and mPGES-1 but not COX-1 and mPGES-1 efficiently couple to form PGE2 from endogenously mobilized AA in intact cells. These findings in combination with a previous observation that cytosolic (c) PLA2γ undergoes a Ca2+-dependent translocation to the Golgi (46) led us to conclude that there is a COX-2-dependent PGE2 biosynthetic system located in the Golgi apparatus.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Cell culture media (DMEM, Opti-MEM, Recover-Cell Culture Freezing Medium, and Lipofectamine 3000), reagents (blasticidin S, hygromycin, phosphate-buffered saline (PBS), TrypLE Express, Golgi-GFP, and pOG44), and supplements (fetal bovine serum (FBS) and goat serum) were obtained from Invitrogen. Tetracycline, cycloheximide, puromycin, tunicamycin, and phosphor 12-myristate 13-acetate were purchased from Sigma. MG132 was obtained from EMD Millipore. H-89 and kifunensine were from Tocris Bioscience. Pyrrophone, a cPLA2γ inhibitor, was from Cayman Chemical Co. Complete protease inhibitor, endoglycosidase H, and peptide-N-glyco- side F were from Roche Applied Science. Restriction enzymes were from New England Biolabs. Rabbit polyclonal anti-cPLA2γ antibody was purchased from Genetex (GTX117583), mouse monoclonal anti-mPGES-1 and anti-COX-2 antibodies were from Cayman Chemical Co. (10004350 and 160112, respectively), mouse monoclonal anti-COX-1 antibody was from Invitrogen (35–8100), mouse monoclonal anti-actin antibody was from MP Biomedical (8691001), and rabbit polyclonal anti-VSV-G tag antibody was from Bio-Rad (AHPL228). VSV-G-
tagged Sar1 and dominant negative Sar1 (H79G) were gifts from Dr. Terry Hebert (McGill University).

Construction of COX Mutant Variants—Most constructs including those derived through site-directed mutagenesis, motif replacements, truncations, or deletions were generated according to the instructions of the QuikChange site-directed mutagenesis kit (Stratagene). The tetracycline-inducible vectors pCDNA5/FRT/TO harboring huCOX-1, huCOX-2, or ovine (ov) COX-1 (29) were used as templates for PCR. In constructing Golgi-ΔSTEL huCOX-1 and Golgi-ΔSTEL huCOX-2, the DNA fragments of Golgi-ΔSTEL huCOX-2 were created from the DNA templates for Golgi-YFP and ΔSTEL huCOX-1 or ΔSTEL huCOX-2 by overlapping extension PCR and then subcloned into pcDNA5/FRT/TO using NotI sites. The correct sequences of constructs were confirmed by DNA sequencing by the University of Michigan DNA Sequencing Core.

Internal ribosome entry sites (IRESs) are sequences that when inserted between the coding sequences for two proteins facilitate co-expression of the two proteins from a single mRNA (47). To generate cell lines co-expressing a COX variant (i.e. huCOX-1, Golgi-ΔSTEL huCOX-1, huCOX-2, KDEL huCOX-2, or Golgi-ΔSTEL huCOX-2) in combination with human mPGES-1, we prepared COX-IRES-mPGES-1 constructs by inserting an IRES-mPGES-1 DNA fragment into an appropriate pCDNA5/COX-variant construct (Invitrogen) using the XhoI site. IRES-mPGES-1 DNA fragments were generated by using overlapping PCR. Briefly, IRES fragments (Fr-A) were obtained from pIRES2-EGFP (Invitrogen) using primers 1 and 2 (below), and the fragments (Fr-B) of the human mPGES-1 gene purchased from Addgene (Plasmid 16506: pBK-CMVgig12) were amplified using primers 3 and 4 (below). Afterward, the IRES-mPGES-1 DNA fragments were obtained using an extension reaction joining Fr-A to Fr-B and a sequential amplification reaction by adding primer 1 and primer 4. During the process, the two XhoI sites highlighted in primers 1 and 4 were introduced. Primer sequences were as follows: primer 1, AATTCTGGCATCGAGGGTACCAGCGG; primer 2, GCTGTGGGCAAGGATGTTGATGCC; primer 3, GGCCACAACCAGGCCCTGCACCA; primer 4, GAAGGTTGTGGCC; primer 3, GGCCACAACCAGGCCCTGCACCA; primer 4, GAAGGTTGTGGCC.

Generation of Stable HEK293 Cell Lines—HEK293-derived cell lines stably and inducibly expressing various COX constructs were generated in tetracycline-inducible mammalian expression systems according to the manufacturer’s instructions (Flip-inR T-RexR, Invitrogen). Briefly, transfections of HEK293 cells in 60-mm cell culture dishes were performed using a calcium phosphate method with various COX pCDNA5 vectors (2 μg) and pOG44 helper vectors (2 μg). After transfection overnight, the cells were trypsinized, collected, and transferred to complete DMEM (i.e. DMEM with 10% FBS) in the presence of 20 μM blasticidin S and 50 μM hygromycin for 2 weeks. Media containing the same amount of antibiotics for selection of clones were changed once after 1-week incubation. The surviving cells were collected and used as stable cell lines.

Stable lines of HEK293 cells inducibly expressing COX-IRES-mPGES-1 were generated as described above except that stable cell lines harboring the various COX-IRES-mPGES-1 sequences were cloned. After an initial 2-week incubation with blasticidin S and hygromycin, surviving single clones were hand-picked and seeded into 12-well culture dishes in complete DMEM containing blasticidin S and hygromycin. Clones expressing both a COX and mPGES-1 robustly were identified by Western blotting. Selected clones capable of inducibly expressing huCOX-1-IRES-mPGES-1, Golgi-ΔSTEL huCOX-1-IRES-mPGES-1, huCOX-2-IRES-mPGES-1, KDEL huCOX-2-IRES-mPGES-1, or Golgi-ΔSTEL huCOX-2-IRES-mPGES-1 were then amplified and stored.

Measurements of COX Protein Degradation—To measure rates of protein degradation of huCOX-2 variants, HEK293 cells stably expressing each of the variants were grown to ~80% confluence, subjected to serum starvation for 24 h, and then treated with tetracycline (10 μg/ml) for another 24 h in complete DMEM to induce the expression of COXs. Protein degradation experiments were then initiated by adding puromycin (50 μg/ml) in the presence or absence of various trafficking inhibitors including MG132 (20 μM) and H-89 (20 μM) and/or other agents. Cells were harvested at various times, and COX and actin protein levels were determined by Western transfer blotting as described in the next section. In decay experiments for Golgi-ΔSTEL huCOX-2 and ΔSTEL huCOX-2, the cells were pretreated with H-89 and MG132 for 1 h and then treated with puromycin (50 μg/ml) in the presence of the same concentrations of inhibitors.

To measure rates of degradation of murine (mu) COX-2, murine NIH 3T3 fibroblasts were first subjected to serum starvation for 48 h and then treated with phorbol 12-myristate 13-acetate (1 μM) for 4 h to stimulate the expression of muCOX-2. Before performing the protein degradation experiments, the cells were preincubated in the presence or absence of various inhibitors (MG132 and H-89) for 1 h at the concentrations described above for studies of huCOX-2 degradation. The degradation experiments with NIH 3T3 cells were then conducted for various times with cycloheximide (50 μM) in the presence or absence of inhibitors.

At the indicated times after different treatments, HEK293 cells or NIH 3T3 cells were harvested and resuspended in ice-cold PBS, pH 7.4 containing Complete protease inhibitor. After sonication, the protein concentration of the whole cell lysate was determined using a BCA protein assay kit (Thermo Scientific). The same amounts of protein sample were loaded into individual lanes for SDS-PAGE and resolved on a 7% tris acetate polyacrylamide gel based on the NuPAGE system (Invitrogen). After transfer to nitrocellulose membranes, the membranes were blocked in Tris-buffered saline with Tween 20 (i.e. 137 mM NaCl, 20 mM Tris-HCl, pH 7.6) for 4 h at 4°C, followed by exposure to x-ray film. Densitometry was performed using ImageJ software.

For Western blotting of native huCOX-2 and huCOX-2-derived mutants, either a rabbit polyclonal antibody previously raised against a peptide extending from Pro-583 to Asn-594 of COX-2 in the Golgi Apparatus
huCOX-2 (29) or a commercial rabbit polyclonal antibody (Novus Biologicals, NB100-689) was used as the primary antibody. For Western blotting of samples from murine NIH 3T3 cells, only the commercial antibody from Novus Biologicals was used as the primary antibody. A rabbit polyclonal antibody raised against a peptide extending from Leu-272 to Gln-283 of ovCOX-1 was used to detect huCOX-1, ovCOX-1, and its mutants (29). A commercial anti-actin antibody raised in mouse was used to detect actin, which was used as the internal control.

Deglycosylation with Endoglycosidase H and Peptide-N-glycosidase F—For deglycosylation of COXs, HEK293 whole cell lysates (3 μg of total protein) were heated at 100 °C in denaturation buffer for 10 min and then treated with endoglycosidase H and/or peptide-N-glycosidase F at 37 °C for 3 h. Afterward, SDS loading buffer was added to the reaction mixture for resolving the proteins by SDS-PAGE. Images were obtained after immunoblotting.

Cell Transfection with Sar1 and H79G Sar1—Transfections of HEK293 cells with Sar1 or dominant negative H79G Sar1 were performed using Lipofectamine 3000 according to the instructions of the manufacturer (Invitrogen). Briefly, 2 × 10⁶ HEK293 cells stably expressing native huCOX-2 were seeded into 6-well cell culture dishes 1 day before transfection. In the process of transfection, a preincubated mixture containing 250 μl of Opti-MEM, 1 μg of a DNA, 2 μl of P3000 reagent, and 350 μl of Lipofectamine 3000 was added dropwise to each well. After a 24-h incubation, the transfection reagent mixtures were removed, and the cells were incubated with tetracycline (10 μg/ml) for another 24 h in fresh complete DMEM to induce COX-2 expression. Finally, protein degradation experiments were conducted by incubating the cells with puromycin (50 μg/ml) for the indicated times, and COX-2 protein levels were determined by immunoblotting.

Immunocytofluorescence—HEK293 cell lines stably and inducibly expressing different COX variants were seeded on four-chambered slides in complete DMEM. The next day, the cells were subjected to a 24-h serum starvation regimen, and then COX expression was induced with tetracycline (10 μg/ml) for 24 h in complete medium. For studies of murine NIH 3T3 fibroblasts and human dermal fibroblasts, the cells were seeded on four-chambered slides, subjected to serum starvation for 48 h, and then stimulated with phorbol 12-myristate 13-acetate (1 μM) with or without 10% FBS for 4 h. For huCOX-1 in human CCL210 (ATCC) lung fibroblasts, after seeding the chambered slides and culturing for 1 day, the cells were used without further treatment. The remaining parts of the protocol were the same for all the immunocytofluorescence studies. After washing with PBS three times, the cells were fixed and permeabilized with 100% methanol at room temperature for 5 min. Prior to immunostaining, the fixed cells were rehydrated and then blocked with PBS containing 0.1% Triton X-100 supplemented with 10% goat serum. Two steps of co-immunostaining were then performed. First, the cells were co-stained with two primary antibodies against a COX and a cellular organelle, respectively. The primary antibodies used were as follows: rabbit polyclonal anti-calnexin (GeneScript), rabbit polyclonal anti-giantin (Abcam), mouse monoclonal anti-COX-2 (Cayman Chemical Co.), and mouse monoclonal anti-COX-1 (Invitrogen) antibodies. In the second step, both Oregon Green anti-rabbit and Texas Red anti-mouse antibodies (Invitrogen) were used as secondary antibodies. After each step of immunostaining, three extensive washes with PBS were performed. Finally, the slides were dried and mounted using a Prolong antifade kit (Invitrogen). Microscopy was conducted in the University of Michigan Center for Live Cell Imaging on a Nikon Infinity confocal microscope at a magnification of 100×. The resultant images were further processed and analyzed with Metamorph software to obtain correlation coefficient (CC) values. CC values for each COX sample were mean values collected from at least 15 cells. To assure a random selection of images, a second, control set of CC values was obtained by rotating one image from one camera 90° and testing for overlap with the image from the other camera; the differences between the observed and random control CC values were statistically different in all cases based on Student’s t test (p < 0.05).

Cyclooxygenase Assays—COX assays were typically conducted in 100 mM Tris-HCl, pH 8.0 containing [1-14C]AA (18 μM; 55 mCi/mmol; American Radiolabeled Chemicals), 5 μM hematin, and 1 mM phenol in the presence or absence of flurbiprofen (500 μM). The reactions were initiated by adding cell lysates to a total volume of 100 μl. After a 0.5–5.0 min incubation at 37 °C, the reactions were quenched with 100 μl of stop solution consisting of ethyl acetate/acetonic acid (95:5, v/v). The resultant samples were centrifuged for 3 min at 4000 × g. An aliquot of the organic layer (~50 μl) was loaded onto a Luna C18(2) column (5 μm; 250 × 4.6 mm; Phenomenex) mounted on a Shimadzu HPLC system equipped with a radio-HPLC detector (IN/US system, β-RAM model 4). The products were analyzed in a gradient elution mode eluting with Solution A (acetoni nitrile/water/acetic acid, 30:70:0.1) for 5 min and then with a linear gradient of Solution A and Solution B (acetoni nitrile/water/acetic acid, 90:10:0.1) for 15 min, then Solution B for 8 min, and finally Solution A for 4 min. The flow rate was 1 ml/min. PGE₂ eluted between 10 and 15 min.

PGE₂ Formation by HEK293 Cells Inducibly Expressing Various huCOX-1ER-ES-mPGES-1 Variants—HEK293 cell lines inducibly expressing COX-1ES-mPGES-1 variants and a sham-transfected HEK cell line were subjected to serum starvation for 24 h and then tetracycline treatment for 24 h to induce formation of a COX variant plus mPGES-1. Both treatments were performed in the presence of 0.25–1 μM AA. The cells were then treated for 1 h with 20 μM MG132 and then with 2 μM A23187 for 45 min or with 12 μM AA for 10 min. The supernatants were collected, and PGE₂ was quantified using a PGE₂ immunoassay kit (Cayman Chemical Co.) or a standard RIA using an anti-PGE₂ antibody from Sigma. All samples were stored at −80 °C before RIAs were performed. Cell samples were collected for BCA protein assays and Western blotting.

RESULTS

Role of the C-terminal STEL Sequence in the Degradation of huCOX-2—Except for ovCOX-1, all mammalian COXs sequenced to date contain a STEL sequence at their C termini (Fig.
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1A) (7); ovCOX-1 has a PTEL sequence. The STEL sequence is a variant of the more typical, robust KDEL ER retention signal found in most mammalian ER-resident proteins. KDEL receptors localized in the cis-Golgi efficiently recognize and bind C-terminal KDEL-like motifs and rapidly transport proteins bearing this motif back to the ER. In contrast, studies of various KDEL-like sequences in HeLa cells indicate that proteins carrying a STEL ER retention sequence are inefficiently transported from the Golgi to the ER (45).

We observed that replacing STEL in huCOX-2 yields a form of the enzyme that is much more stable (t1/2 > 24 h) than native huCOX-2 (t1/2 < 0.5 h) (Fig. 1, A–C). Thus, insertion of

![Diagram](image-url)

**FIGURE 1.** Domain structures and degradation of huCOX-2 constructs targeted to different organelles. A, domain structures of huCOX-2, KDEL huCOX-2, and Golgi-ΔSTEL huCOX-2 constructs indicating known sites of N-glycosylation. The signal peptide of huCOX-2 was replaced with the N terminus of β-1,4-galactosyltransferase in preparing Golgi-ΔSTEL huCOX-2. These various constructs and homologs having N594A substitutions were prepared and used for stable, tetracycline-inducible expression in HEK293 cells as described under “Experimental Procedures.” SP, signal peptide; EGF, EGF domain; MBD, membrane binding domain; CatD, catalytic domain. The sequences of the C-terminal ER retention signal of the constructs differ as indicated. B, degradation of different huCOX-2 variants and their N594A homologs. HEK293 cells were cultured to express the indicated variants, and disappearance of COX-2 with time following the addition of puromycin was monitored by Western transfer blotting as detailed under “Experimental Procedures.” The two arrows shown next to the blot for KDEL huCOX-2 denote the two glycosylated forms of protein and the time-dependent increase of the slower mobility species. The primary antibody for all the Western blots shown in this figure was a rabbit anti-huCOX-2 antibody prepared against a peptide with the sequence from Pro-583 to Asn-594 of huCOX-2 (29). Experiments were repeated at least three times with similar results. C, densitometric measurement of COX-2 degradation. Densitometry was performed as described under “Experimental Procedures” using ImageJ software. Data are shown as mean ± S.D. (error bars). Based on repeat measure of ANOVA (IBM SPSS Statistics 21), the rate of degradation between huCOX-2 and N594A huCOX-2 and between Golgi-ΔSTEL huCOX-2 and N594A Golgi-ΔSTEL huCOX-2 are significantly different (p < 0.05). The differences are denoted by an asterisk in each case. N594A huCOX-2, KDEL huCOX-2, and N594A KDEL huCOX-2 are not degraded at appreciable rates. D, effect of tunicamycin on the degradation of KDEL huCOX-2. COX-2 degradation was measured essentially as described for B and C above. Tunicamycin at the indicated concentrations was added to the medium at the same time as puromycin, and cells were harvested at either 0, 8, or 24 h following the addition of puromycin. The fractions of COX-2 in the upper and lower bands of KDEL huCOX-2 observed 24 h following the addition of tunicamycin and puromycin were determined by densitometry and are shown in the lower panel. E, effect of inhibitors of lysosomal degradation on the degradation of native huCOX-2. Experiments were performed essentially as described in B above but in the presence of the indicated concentrations of inhibitors of lysosomal proteolysis. Tet, tetracycline.
a strong ER retention signal slows the degradation of huCOX-2. The impact of the KDEL substitution on huCOX-2 degradation is comparable with that of an N594A substitution or deletion or modifications of certain other residues in the C-terminal degron of huCOX-2 (28, 29). As shown in Fig. 1B, inhibition of KDEL huCOX-2 protein synthesis by puromycin leads to a time-dependent accumulation of a more slowly migrating form of the enzyme; when treated with endoglycosidase H and then subjected to Western blotting, only a single band (M, ~ 65,000; data not shown) was observed, indicating that the slower mobility species seen with KDEL huCOX-2 in Fig. 1B resulted from post-translational changes in N-glycosylation.

Based on previous studies of the degradation of native COX-2 in the presence of puromycin plus the α-1,2-mannosidase inhibitor kifunensine (29), we suspected that the time-dependent formation of the upper band seen with KDEL huCOX-2 was due to N-glycosylation of Asn-594. As a test of this concept, we analyzed an N594A KDEL huCOX-2. This mutant was also degradation-resistant, but no time-dependent accumulation of a less mobile form was apparent. Finally, we found that the formation of the slowly moving species observed with KDEL huCOX-2 was retarded by tunicamycin (≥5 μM), which by inhibiting N-acetylglucosamine-1-phosphate transferase retards N-glycosylation (48) (Fig. 1D). These results indicate that the less mobile form of KDEL huCOX-2 results from the N-glycosylation of Asn-594. The results also establish that in the case of KDEL huCOX-2 Asn-594 glycosylation and proteasomal degradation are not directly coupled. This is consistent with our previous conclusion that glycosylation of Asn-594 is necessary but not sufficient to enable proteasomal degradation of COX-2 (29). The present results and those reported previously (29) also suggest that glycosylation of Asn-594 of both native and KDEL huCOX-2 occur in the ER lumen in a relatively slow manner. Importantly, restricting the trafficking of huCOX-2 by introducing a strong ER retention signal markedly slows or prevents the degradation of huCOX-2 via the ERAD pathway.

Another mutant denoted Golgi-ΔSTEL huCOX-2 (Fig. 1A) was engineered to examine the effects of protein localization on the degradation of huCOX-2. β-1,4-Galactosyltransferase consists of a short N-terminal cytoplasmic domain, a transmembrane domain as well as a stem region, and a C-terminal catalytic domain that localize to the lumen side of Golgi complex. The intact transmembrane domain is responsible for its subcellular localization to the medial- and trans-Golgi complex (49).

As shown in Fig. 1A, we replaced the catalytic domain of β-1,4-galactosyltransferase with the huCOX-2 sequence lacking both its signal peptide and C-terminal STEL sequence. This construct was degraded much more rapidly (t1/2 ~ 30 min) than native huCOX-2 (Fig. 1B and C). As discussed below, a proteasome inhibitor (MG132) and lysosomal inhibitors (chloroquine and NH4Cl) attenuated the degradation of Golgi-ΔSTEL huCOX-2. This suggests that the degradation of Golgi-ΔSTEL huCOX-2 involving the proteasome may not involve the same route as that of native huCOX-2. Degradation of native huCOX-2 in HEK293 cells was not inhibited either by the lysosomal cysteine protease inhibitor E-64 or by NH4Cl or chloroquine treatments (Fig. 1E) consistent with previous results indicating that native huCOX-2 does not undergo lysosomal degradation (28, 29).

The N594A Golgi-ΔSTEL huCOX-2 variant was even less stable than Golgi-ΔSTEL huCOX-2 (Fig. 1, B and C). ΔSTEL huCOX-2 was somewhat less stable (t1/2 ~ 3 h) (data not shown) than native huCOX-2 (t1/2 ~ 5 h). As discussed below, we suspect that this is because ΔSTEL huCOX-2 enters the secretory pathway. Similarly, we speculate that the pronounced instability of N594A Golgi-ΔSTEL huCOX-2 is because this variant enters the secretory pathway.

The inhibitory effects on huCOX-2 degradation in HEK293 cells by trafficking inhibitors were dose-dependent (data not shown). The effective concentration for H-89 was 20 μM, which is about 3 orders of magnitude greater than the Ki value of H-89 with PKA (0.048 μM) (51), suggesting that the target(s) of H-89 in this process is not PKA. These pharmacologic data suggest that the trafficking of huCOX-2 in HEK293 cells is essential for its efficient degradation via the ERAD pathway and that both COPI- and COPII-coated vesicles are involved in the trafficking of huCOX-2.

Next, we examined the effects of inhibitors on degradation of endogenously expressed muCOX-2 in NIH 3T3 cells using a commercial antibody (Novus Biologicals). The results clearly show that H-89 and MG132 block the degradation of muCOX-2 (Fig. 2B).

We also tested the effect of H-89 on the degradation of KDEL huCOX-2 and Golgi-ΔSTEL huCOX-2 expressed heterologously in HEK293 cells. H-89 did not affect the degradation of KDEL huCOX-2 (data not shown). Similarly to what was observed with native huCOX-2, MG132 partially stabilized Golgi-ΔSTEL huCOX-2 as did the lysosomal inhibitors chloroquine and NH4Cl (data not shown). These latter results suggest that Golgi-ΔSTEL huCOX-2 is a substrate for both proteasomal and lysosomal degradation. In summary, evidence from
studies with several relatively nonspecific protein trafficking inhibitors indicates that organellar trafficking is involved in the degradation of COX-2.

 Effects of a Dominant Negative Sar1 on COX-2 Protein Degradation—As an additional test of the role of protein trafficking in COX-2 degradation, we examined the degradation of hUCOX-2 in HEK293 cells transiently transfected with the dominant negative H79G Sar1 mutant. The core layer of COPII involved in anterograde transport consists of Sar1 and Sec23-24 and Sec13-31 complexes. These are sequentially recruited to the ER membrane to form COPII vesicles. Sar1, a small GTPase, plays a central role in assembling and disassembling the COPII coats. In the studies depicted in Fig. 3, transiently expressed VSV-G-tagged Sar1 and H79G Sar1 were examined for their abilities to affect COX-2 degradation. Dominant negative H79G Sar1 is a constitutively active, GTP-bound form of Sar1. Although efficiently recruited to ER membrane to form COPII vesicles, the inability of H79G Sar1 to hydrolyze GTP hampers the disassembly of COPII vesicles. This results in inhibition of ER to Golgi transport and disruption of ERGIC and Golgi compartments (53). Compared with the control experiment with empty plasmid, overexpression of native Sar1 had relatively little effect on COX-2 degradation (Fig. 3). More than 60% of the hUCOX-2 was degraded within 8 h after transfection with vector or native Sar1. There was slightly less COX-2 at 0 h in cells treated with native Sar1, suggesting that excess Sar1 can promote COX-2 degradation. In cells treated with dominant negative H79G Sar1, the rate of hUCOX-2 degradation was slowed significantly, and about 80% of the hUCOX-2 remained after 8 h. Similar levels of VSV-G-labeled Sar1 and VSV-G-labeled H79G Sar1 were expressed under the conditions of the experiment. This experiment indicates that disrupting protein anterograde ER to Golgi transport affects the degradation of hUCOX-2 occurring via the ERAD pathway and that trafficking of hUCOX-2 utilizes the COPII coat system.

FIGURE 2. Protein trafficking inhibitors attenuate the degradation of hUCOX-2 via the ERAD pathway. A, effects of inhibitors on the degradation of hUCOX-2 heterologously expressed in HEK293 cells. Degradation experiments were performed as described under “Experimental Procedures” and the legend to Fig. 1B. For the experiments shown in this panel, puromycin was added to all the samples (i.e. both in the presence and absence of trafficking inhibitors). The following inhibitors were tested: MG132 (20 μM), H-89 (20 μM), and AlF4⁻ (50 μM AlCl₃ plus 30 mM NaF). Data are shown as mean ± S.D. (error bars). Based on repeated measures of ANOVA (IBM SPSS Statistics 21), the differences between the control group without drug treatment and the treated groups except for AlF4⁻ are statistically significant (p < 0.05). These differences are denoted with asterisks. B, effects of inhibitors on the degradation of endogenous native muCOX-2 in murine NIH 3T3 fibroblasts. NIH 3T3 cells were cultured to express COX-2 as detailed under “Experimental Procedures.” After the cells were treated with inhibitors for 1 h, cycloheximide (50 μM) was added to block translation along with the amounts of inhibitors indicated in A above. Cells were collected at the indicated times, and proteins were subjected to Western blotting. The densitometry measurements of muCOX-2 are based on two separate experiments. The primary antibody used for the experiments with murine NIH 3T3 cells was a commercial antibody from Novus Biologicals. Data are shown as mean ± S.D. (error bars). Based on repeated measures of ANOVA (IBM SPSS Statistics 21), the differences between the control group without drug treatment and the MG132- and H-89-treated groups are statistically significant (p < 0.05) from 0 to 24 h. These differences are denoted with asterisks.
performed parallel studies with several fibroblast lines that endogenously express COX-1 and/or COX-2 and in which the subcellular structures appear in better definition.

As shown in Fig. 4A, image overlay data indicate that a part of native huCOX-2 expressed heterologously in HEK293 cells is localized in the Golgi apparatus. Similarly, in murine NIH 3T3 fibroblasts, part of the endogenous muCOX-2 also co-localized with giantin closely neighboring the nucleus; the pattern of intense perinuclear staining is quite similar to that observed previously using normal phase immunocytofluorescence (24). Golgi-ΔSTEL huCOX-2 in HEK293 cells was also largely if not exclusively co-localized with giantin, whereas neither KDEL huCOX-2 nor N594A huCOX-2 co-localized with giantin (Fig. 4A). The distribution of huCOX-1 and ovCOX-1 expressed in HEK cells and endogenous huCOX-1 in CCL210 cells was also investigated by confocal microscopy (Fig. 4B). The COX-1 forms examined did not exhibit significant overlap with giantin but did overlap with the ER marker calnexin. We emphasize that the majority of both COX-1 and COX-2 staining was associated with the ER and nuclear envelope consistent with much previous work (19–23). This is illustrated in the bottom panel of Fig. 4B that shows widespread staining throughout the cell with considerable overlap between calnexin staining and ovCOX-1 staining in HEK293 cells.

CCs were determined for giantin staining and the staining of various forms of endogenous and heterologously expressed COX-1 and COX-2 (Fig. 4C). Native muCOX-2 in 3T3 cells and native huCOX-2, Golgi-ΔSTEL huCOX-2, and KDEL huCOX-2 in HEK293 cells exhibited CCs of 0.33, 0.31, 0.42, and −0.10, respectively (Fig. 4C). These data indicate that there is a significant difference between the subcellular locations of native huCOX-2 and Golgi-ΔSTEL huCOX-2 compared with KDEL huCOX-2 in HEK cells and that native huCOX-2 expressed heterologously and native muCOX-2 expressed endogenously were similarly localized. ΔSTEL huCOX-2 with a CC of 0.09 does not closely co-localize with the giantin marker. However, only a low level of fluorescent staining was observed with this variant. We suspect that ΔSTEL huCOX-2 can move to the Golgi but, lacking both an ER retention signal and the Golgi anchoring sequence of Golgi-ΔSTEL huCOX-2, then moves on through the secretory pathway. CCs for the Golgi marker with ovCOX-1 and huCOX-1 in HEK cells were 0.03 and 0.05, respectively, whereas the value for endogenously expressed huCOX-1 in CCL210 cells was 0.02. Again, these numbers are significantly lower for COX-1 than those for native huCOX-2 and muCOX-2 and occurred across several cell types with heterologously expressed and endogenous COX-1.

Interestingly, the CC for N594A huCOX-2 and giantin in HEK293 cells is 0.06, indicating that N594A huCOX-2 is not localized in the Golgi. This observation indicates that post-translational glycosylation of Asn-594 occurs in the ER lumen, that glycosylated Asn-594 is an ER exit signal, and that variant forms of COX-2 having mutations that prevent N-glycosylation of Asn-594 become lodged in the ER. Overall, our confocal microscopy data support the hypothesis that a significant fraction of COX-2 but not COX-1 resides in the Golgi apparatus.

Subcellular Location of COXs and mPGES-1 as Determined by Confocal Microscopy—To examine the concept that COX-2 can migrate to and reside in the Golgi apparatus, the subcellular location of COX-2 was investigated by confocal fluorescence microscopy. Giantin, a highly conserved cis- and medial-Golgi-resident protein, was used as the Golgi marker, and calnexin was used as the ER marker. For studies with HEK293 cells, we tested COX-2 mutants that we expected to be primarily localized to either the ER or the Golgi for comparison. HEK293 cells are not ideal for immunocytofluorescence studies, and so we

FIGURE 3. Dominant negative H79G Sar1 attenuates the degradation of huCOX-2 via the ERAD pathway. A, Western transfer blots from experiments to determine the effect of Sar1 variants on the degradation of huCOX-2 expressed heterologously in HEK293 cells. As described under “Experimental Procedures,” HEK293 cells stably expressing native huCOX-2 were (a) transfected with pCDNA-3, Sar1-pCDNA-3, or H79G Sar1-pCDNA-3; (b) treated with tetracycline to induce COX-2; and (c) incubated with puromycin for 0 or 8 h. COX-2, VSV-G-tagged Sar1, and actin protein levels at 0 and 8 h were then analyzed by Western blotting. B, the relative amounts of immunoreactive huCOX-2 protein normalized to COX-2 density in the pCDNA-3 cells at the 0-h puromycin time point. Values were determined by densitometry of Western blots and represent mean values ± S.D. (error bars) from three independent experiments. Tukey’s multiple comparison test was used to determine statistically significant differences in densities (p < 0.05) and are indicated in the figure as follows: a, value different from zero time value for pCDNA3; b, value different from zero time value for Sar1-pCDNA3; and c, value different from 8-h value for pCDNA-3 and 8-h value for Sar1-pCDNA-3. There was no statistically significant difference between the COX-2 levels at 0 h in cells treated with pCDNA3 versus H79G Sar1-pCDNA3.

COX-2 in the Golgi Apparatus

A.

B.
We speculate that because COX-1 lacks an efficient ER to Golgi trafficking signal (i.e. glycosylated Asn-594) this isoform resides primarily in ER.

Finally, we examined the subcellular location of human mPGES-1 expressed heterologously in HEK293 cells and endogenous mPGES-1 in human dermal fibroblasts (Fig. 4D).

**FIGURE 4.** Subcellular localization by confocal fluorescence microscopy of COX-1 and COX-2 variants and mPGES-1. A, co-localization with the Golgi marker giantin of various huCOX-2 variants expressed heterologously in HEK293 cells and endogenous muCOX-2 expressed in NIH 3T3 fibroblasts. HEK293 cell lines stably and inducibly expressing different huCOX-2 variants were cultured in four-chambered slides and subjected to immunocytofluorescence as detailed under “Experimental Procedures.” The primary antibodies used were rabbit polyclonal anti-giantin antibody and murine monoclonal anti-COX-2 antibody. Microscopy was conducted on a Nikon Infinity confocal microscope at a magnification of 100X. B, co-localization of huCOX-1 in HEK293 cells, ovCOX-1 in HEK293 cells, and huCOX-1 in CCL210 human lung fibroblasts with Golgi (giantin) and ER (calnexin) markers. For CCL210 cells, the slides were directly fixed and prepared 1 day after seeding the cells. The protocols used for staining the HEK293 cells were as described in A above. The primary antibodies used were mouse monoclonal anti-COX-1 and either rabbit polyclonal anti-giantin or rabbit polyclonal anti-calnexin. C, CC values for co-localization of different COX-1 and COX-2 variants with the Golgi marker giantin were determined as detailed under “Experimental Procedures”. Based on the one-way ANOVA for multiple comparisons (GraphPad Prism), a difference between CC values for huCOX-2 in HEK293 cells, muCOX-2 in 3T3 cells, and Golgi-ΔSTEL huCOX-2 in HEK293 cells and the CC values for COX-1 and other COX-2 variants are statistically significant (*p* < 0.05) as denoted with asterisks (*). No significant difference was observed between huCOX-2 in HEK293 cells and muCOX-2 in 3T3 cells. D, co-localization of mPGES-1 with the Golgi marker giantin and the ER marker calnexin in human dermal fibroblasts (HDFn). Human dermal fibroblasts were cultured to express endogenous huCOX-2, and immunocytofluorescence was performed as described under “Experimental Procedures”. A mouse monoclonal antibody to mPGES-1 was used as the primary antibody.
The patterns of staining are similar to those seen for huCOX-2 in HEK293 cells and mCOX-2 in NIH 3T3 cells.

Specific Activities of Native huCOX-2, KDEL huCOX-2, and Golgi-STEI huCOX-2—Although COX-2 protein was present in different organelles, it was not clear whether the mutant COX-2 variants were functional. To address this question, COX assays were performed by incubating 18 μM [1-14C]AA with lysates prepared from HEK293 cells expressing native huCOX-2, KDEL huCOX-2, or Golgi-DESTEI huCOX-2. These particular HEK293 cell lines have very low levels of mPGES-1. After formation via COX activity, PGH2 rearranges non-enzymatically to a number of products. These products were separated by HPLC and quantified by scintillation counting. PGH2-derived products (i.e. PGs and the mono-oxygenated product 17-hydroxyeicosatetraenoic acid) were generated by lysates prepared from each cell line, and product formation was inhibited by ibuprofen, a common COX inhibitor. COX-2 protein levels in the lysates were estimated using Western blotting and densitometry comparing the staining intensities of the COX-2 in cell lysates with those of known amounts of purified recombinant huCOX-2 (16). Specific activities for huCOX-2, KDEL huCOX-2, and Golgi-DESTEI huCOX-2 were 13, 12, and 17 μmol of AA consumed/min/mg of COX-reactive protein, respectively. These values, which were determined with 18 μM AA, agree reasonably well with the Vmax value of ~21 μmol AA/min/mg determined for purified, recombinant huCOX-2 given that the Km for purified huCOX-2 is ~10 μM (16). Importantly, Golgi-DESTEI huCOX-2, most if not all of which appears to be localized to the Golgi apparatus (Fig. 4A), exhibited high levels of COX activity. Our quantitative measures of COX-2 activity and protein indicate that the membrane environment of the different organelles does not change the overall structure of COXs and importantly that COX-2 present in the Golgi apparatus is functional.

Metabolic Coupling of COX-1 and COX-2 Variants with cPLA2a and mPGES-1—We next performed a set of measurements with intact cells to test for metabolic coupling of cPLA2a, COX-1 and COX-2, and mPGES-1 in the ER versus the Golgi apparatus (Fig. 5). Stable HEK293 cell lines inducibly expressing huCOX-1, Golgi-DESTEI huCOX-1, huCOX-2, KDEL huCOX-2, or Golgi-DESTEI huCOX-2 in combination with human mPGES-1 were prepared. In developing these HEK293 cell lines, we used recombinant constructs having an IRES linker (47) between the coding regions of the COX variant and the mPGES-1; this linker promotes co-translation of the indicated COX with mPGES-1. These lines are denoted as huCOX-1-IRES-mPGES-1, Golgi-DESTEI huCOX-1-IRES-mPGES-1, huCOX-2-IRES-mPGES-1, KDEL huCOX-2-IRES-mPGES-1, or Golgi-DESTEI huCOX-2-IRES-mPGES-1 as described under “Experimental Procedures”. Cultured cells from each of these lines and from a sham-transfected HEK cell line were subjected to serum starvation for 24 h and then tetracycline treatment for 24 h to induce formation of a COX variant plus mPGES-1; both treatments were performed in the presence of 0.25–1 μM AA. The cells were then treated for 1 h with 20 μM MG132 to minimize ERAD. A, Western transfer blotting of COX-1 or COX-2 and mPGES-1 was performed as described under “Experimental Procedures” using 12 μg of total cell protein from each of the indicated cell lines. No COX or mPGES-1 immunoreactivity was observed using protein from the sham-transfected cells (not shown). The entire gel was stained for actin and mPGES-1, and segments of the gels were then stained for huCOX-1 or huCOX-2. B, PGE2 formation by HEK293 cells inducibly expressing various huCOX-IRES-mPGES-1 variants. HEK293 cell lines were developed that inducibly express huCOX-1-IRES-mPGES-1, Golgi-DESTEI huCOX-1-IRES-mPGES-1, huCOX-2-IRES-mPGES-1, KDEL huCOX-2-IRES-mPGES-1, or Golgi-DESTEI huCOX-2-IRES-mPGES-1 as described under “Experimental Procedures”. Cultured cells from each of these lines and from a sham-transfected HEK cell line were subjected to serum starvation for 24 h and then tetracycline treatment for 24 h to induce formation of a COX variant plus mPGES-1; both treatments were performed in the presence of 0.25–1 μM AA. The cells were then treated for 1 h with 20 μM MG132 to minimize ERAD. A, Western transfer blotting of COX-1 or COX-2 and mPGES-1 was performed as described under “Experimental Procedures” using 12 μg of total cell protein from each of the indicated cell lines. No COX or mPGES-1 immunoreactivity was observed using protein from the sham-transfected cells (not shown). The entire gel was stained for actin and mPGES-1, and segments of the gels were then stained for huCOX-1 or huCOX-2. B, PGE2 formation by HEK293 cell lines inducibly expressing the COX-IRES-mPGES-1 variants in panel A above. The cell lines were incubated with either 12 μM AA for 10 min or 2 μM A23187 for 45 min, the supernatants were collected, and PGE2 levels were quantified using a PGE2 enzyme immunoassay kit or a standard RIA. Data are from four separate experiments performed at different times with different preparations of the cultured cells. No PGE2 was formed by sham-transfected HEK293 cells (not shown). The ratios of the amounts of PGE2 formed after treatment with A23187 (i.e. from endogenous AA) compared with the amounts of PGE2 formed after treatment with 12 μM AA (×10–3) (i.e. from exogenous AA) are shown under the brackets. Based on the one-way ANOVA for multiple comparisons (GraphPad Prism), the differences in the ratios (A23187/exogenous 12 μM AA) between the huCOX-2-IRES-mPGES-1 and both KDEL huCOX-2-IRES-mPGES-1 and Golgi-DESTEI huCOX-2-IRES-mPGES-1 are statistically significant (p < 0.05); in addition, the differences in the ratios between huCOX-1-IRES-mPGES-1 and both Golgi-DESTEI huCOX-1-IRES-mPGES-1 and huCOX-2-IRES-mPGES-1 are statistically significant (p < 0.05). These statistically significant differences are denoted with asterisks.
in Fig. 5A. We also examined the PG product profiles for each of the five cell lines. When incubated with exogenous [1-14C]AA (12 μM) for 10 min, the only significant PG product in each case was PGE2 as determined using HPLC with PG standards (14) (data not shown). This indicates that there is sufficient mPGES-1 activity in each cell line to convert any COX-derived PGH2 to PGE2. A 10-fold range of COX-specific activities was observed with the different lines (Fig. 5B): KDEL huCOX-2 > huCOX-1 > huCOX-2 > Golgi-ΔSTEL huCOX-2 > Golgi-ΔSTEL huCOX-1. These differences reflect differences in the amounts of catalytically active COX protein because COX-1 and COX-2 have similar kinetic constants with AA as substrate (7).

Finally, we compared PGE2 production by each of the cell lines following treatment with excess exogenous AA (12 μM) versus a relatively low concentration of the Ca2+ ionophore A23187 (2 μM) to mobilize a low concentration of endogenous AA (AA). A23187 elicits mobilization of endogenous AA for PG production by activating Ca2+-dependent PLA2s including cPLA2. Also enumerated in Fig. 5B are the ratios of PGE2 formed from cells treated with 2 μM A23187 versus cells treated with exogenous 12 μM AA (×10−5). Because similar amounts of cPLA2 are present in the cells (Fig. 5A), these values indicate the relative abilities of the different COX forms to generate PGE2 from endogenous AA relative to exogenous AA. Cells expressing mPGES-1 in combination with native huCOX-2, Golgi-ΔSTEL huCOX-2, or Golgi-ΔSTEL huCOX-1 all exhibited significantly higher levels of PGE2 formation from endogenous versus exogenous AA than COX variants that are concentrated in the ER (i.e., huCOX-1 or KDEL huCOX-2). It should be noted that treatment of the cell lines with the COX inhibitor flurbiprofen 1 h prior to either the addition of exogenous 12 μM AA or treatment with 2 μM A23187 caused in each case >95% inhibition of PGE2 formation (data not shown). Moreover, treatment with the cPLA2, inhibitor pyrrophenone 1 h prior to the addition of 2 μM A23187 caused 80% inhibition of PGE2 formation; however and as expected, pyrrophenone did not interfere with PGE2 formation from exogenous AA. The results in Fig. 5 indicate that PGE2 formation from endogenous AA occurs more efficiently with COX variants present in the Golgi apparatus than in the ER. The results suggest that cPLA2, COX-2, and mPGES-1 in the Golgi provide an efficient system for PGE2 formation.

**DISCUSSION**

Overall, the results presented in this report suggest that there is a dedicated system for COX-2-mediated PGE2 synthesis localized in the Golgi apparatus of cells. This system could account for the longstanding enigma of how COX-2 can function under conditions in which COX-1 is relatively inoperative when both COX isoforms are expressed in cells (54). Our conclusions emerged from studies of COX-2 protein degradation, so we first discuss the work in the context of COX-2 as an unusual substrate of ERAD.

Degron-mediated ERAD of COX-2 Involves ER to Golgi to ER Trafficking—The ERAD pathway is often regarded as a quality control mechanism for disposing of misfolded, wrongly glycosylated, or unassembled ER proteins (for recent reviews, see Refs. 36–40). However, ERAD can also play a role in the disposal of correctly folded ER proteins (39). For instance, to avoid detection by the immune system, human cytomegalovirus uses US11 and US2 proteins as adaptors to deliver correctly folded MHC class I heavy chains to the ERAD system (55). Another example is the elegant regulation of 3-hydroxy-3-methylglutaryl-CoA reductase by physiological sterol concentrations. In mammalian cells, high sterol levels elicit structural changes in 3-hydroxy-3-methylglutaryl-CoA reductase, and the then pseudo-unfolded enzyme can bind to adaptor proteins Insig-1 and Insig-2 to promote ERAD (56, 57).

The aforementioned ERAD target proteins are transmembrane proteins. In contrast, COXs are integral membrane proteins that are embedded in a single layer of the lipid bilayer through a membrane binding domain composed of four amphipathic helices (7). A large, soluble catalytic domain extends into the vesicle lumen. The degradation of apparently intact COX-2 via ERAD involves a modular degron located near the C terminus of the catalytic domain (28, 29). COX-2 with its intrinsic degron represents a relatively new category of ERAD substrates in mammalian proteostasis.

Central conclusions of our study are illustrated in Fig. 6, which depicts degron-coupled ERAD of COX-2. Key features of this model are as follows. Newly translated and folded COX-2 is post-translationally glycosylated at Asn-594 in the ER lumen and then deglycosylated by glucosidases I and II to generate an ER departure signal (36, 39), presumably the Man9(GlcNAc)2-Asn-594 moiety (58–61). We propose that this Asn-594-linked oligosaccharide binds to a member of the Ca2+-dependent L-type family of lectins such as ERGIC-53, VIPL (vesicular-integral membrane protein 36-like protein), or VIP-63 in conjunction with an appropriate adapter protein such as MCDF-2 (60), leading in turn to packaging COX-2 into COPII vesicles (36). The structure of the COX-2 ER outgoing signal is not known but presumably involves the Asn-594-linked carbohydrate and perhaps an exposed β-hairpin loop between Val-572 and Thr-588 (29) analogous to that of procathespisin Z (58). COX-2 then undergoes anterograde transport to the Golgi apparatus. Data supporting ER to Golgi trafficking of COX-2 include (a) previous evidence for post-translational N-glycosylation of Asn-594 (28, 29) and now the demonstration that post-translational N-glycosylation of KDEL huCOX-2 occurs in the ER, (b) the inhibition of COX-2 degradation by a dominant negative Sar1 and H-89, and (c) the detection of significant levels of COX-2 in the Golgi by confocal immunofluorescence. This latter result is also consistent with a recent report of COX-2 in the Golgi apparatus visualized using a fluorescent active site probe for COX-2 (62). We envision that the weak ER retention sequence STEL (45) of COX-2 that is present in all mammalian COX-2s sequenced to date (7) provides for a relatively extended residence time in the Golgi. This permits ER mannosidase-1 to remove the Man9(GlcNAc)2-Asn-594 moiety, generating a Man8 (GlcNAc)2-Asn-594 species; we assume that this has no effect on enzyme activity as NS594A COX-2 mutants have the same activity as native COX-2 (42). Eventually, the modified but still catalytically active COX-2 undergoes retrograde transport to the ER via a COPI vesicle in a process inhibited by AlF4−. We speculate that once returned to the ER the Man9(GlcNAc)2-Asn-594 group is trimmed on the C-chain perhaps through the
action of EDEM3 to Man$_7$(GlcNAc)$_2$Asn-594 (38, 44). Finally, in events involving EDEM, ERp57, OS-9, XTP3-B, and the HRD1-SEL1L ubiquitin ligase complex (39, 44), a trimmed and unfolded COX-2 is translocated to the cytosol for ubiquitination and proteasomal degradation (25, 28, 29, 32, 35). The slow glycosylation observed with KDEL huCOX-2 suggests that the rate-limiting step in ERAD of COX-2 is post-translational glycosylation of Asn-594 in the ER. The rate of post-translational N-glycosylation of Asn-594 of COX-2 could be subject to control, which could in turn regulate the rate of both COX-2 ERAD and COX-2-mediated PGE$_2$ formation in the Golgi.

In the model proposed in Fig. 6, the proper folding and post-translational N-glycosylation of COX-2 in the ER is spatially separate from the ER mannosidase-1-mediated event in the Golgi that eventually leads to ERAD. This spatial segregation eliminates competition between COX-2 protein folding and degradation. The model can explain the observation that N-glycosylation of Asn-594 is necessary but not sufficient for ERAD of COX-2 (29). The process differs from that involving co-translational N-glycosylation, which is most commonly associated with glycoprotein degradation via ERAD.

Finally, with respect to degradation, we note that in addition to ERAD COX-2 is subject to substrate turnover-induced degradation (29, 30). This latter process does not require N-glycosylation of Asn-594. We speculate that substrate turnover-induced degradation is a backup degradation system for the COX-2 that accumulates in the ER but is (a) not N-glycosylated for transport to the Golgi for PGE$_2$ synthesis and/or (b) not needed for synthesizing other PG products in the ER. Substrate turnover-induced degradation of COX-2 would be expected to occur in the presence of excess substrate, a condition favoring COX-1 activity. In cells co-expressing both isoforms, COX-2 is typically in much lesser abundance than COX-1 (63).

A Golgi cPLA$_{2\alpha}$/COX-2/mPGES-1 System for PGE$_2$ Synthesis—Reddy and Herschman (54) were the first to demonstrate that when COX-1 and COX-2 are co-expressed in cells that COX-2 can function with endogenously mobilized AA, whereas COX-1 is effectively latent; however, when treated with a bolus of exogenous AA, both COX-1 and COX-2 are operative. Several explanations have been proffered for these observations. One is simply that the effective concentration of AA released endogenously is significantly less than that when cells are treated with a bolus of exogenous AA and that COX-2 has a slightly lower $K_m$ than COX-1 for AA (64). A related possibility is that COX-2 operates within an endogenous pool of non-substrate fatty acids that through allosteric mechanisms favors COX-2 over COX-1 activity (14–17). Additionally or alternatively, COX-2 has a lesser hydroperoxide requirement than COX-1 for its activation (6), and the concentration of activating hydroperoxides present following COX-2 induction may be too low to activate COX-1. These kinetic explanations are difficult to test in intact cells, and there may be other explanations for the preferential use of endogenous AA by COX-2. Our studies suggest one alternative: that COX-1 and COX-2 can operate independently in cells because they function at least in part in geographically separate compartments.

Our results suggest that ER to Golgi transport provides COX-2 to a compartmentalized cPLA$_{2\alpha}$/COX-2/mPGES-1 system for
COX-2 in the Golgi Apparatus

PGE\(_2\) synthesis. We found COX-2 but little or no COX-1 in the Golgi apparatus. The reason is that only COX-2 has an ER to Golgi trafficking signal. Furthermore, we found that mPGES-1 is also located in the Golgi apparatus. Finally, another key enzyme, cPLA\(_2\), which plays a central role in regulating AA release for PGE\(_2\) synthesis in response to intracellular Ca\(^{2+}\) mobilization, binds to Golgi in preference to ER membranes at lower cytosolic Ca\(^{2+}\) concentrations (46). According to our model (Fig. 6), part of the cellular COX-2 resides in the Golgi and would have access to this endogenous pool of AA when it is mobilized by cPLA\(_2\) at low intracellular Ca\(^{2+}\) levels (i.e., when only low levels of endogenous AA are being released). In contrast, COX-1 resides primarily if not exclusively in the ER where AA is not accessible to COX-1 at lower cytosolic Ca\(^{2+}\) concentrations.

PGE\(_2\) formed in the Golgi would be expected to be produced at low levels relative to that produced via a burst of COX-1 action in the ER (54). This is because there is almost always less COX-2 than COX-1 (63), and as noted earlier and discussed further below, most of the COX-2 appears to be in the ER rather than the Golgi. Accordingly, we speculate that PGE\(_2\) produced via COX-2 in the Golgi would appear at low concentrations extracellularly. This would favor autocrine rather than paracrine actions of PGE\(_2\).

We note that there are previous reports that cPLA\(_{2\alpha}\) and COX-2 co-localize with caveolae in fibroblasts (65) and in lipid droplets in many cell types (66). Additionally, there is a significant body of evidence indicating that cPLA\(_{2\alpha}\) and COX-2 function coordinately (67–69). COX-2 and mPGES-1 are also functionally related (for a recent review, see Ref. 7). There is a previous report of perinuclear mPGES-1 immunocytofluorescence in human chondrocytes that partially overlaps with COX-2 fluorescence (68), and this could result from the staining of mPGES-1 in the Golgi.

It is important to recognize that most of the COX-2 staining observed in our studies was present in the ER and not the Golgi apparatus (Fig. 4). Unfortunately, there are no robust methods for quantifying the relative amounts of COX-2 (or cPLA\(_{2\alpha}\) or mPGES-1) in the Golgi versus the ER of cultured cells. COX-2 appears to be only slowly glycosylated on Asn-594, and thus, trafficking from the ER to the Golgi is not very efficient. This is an important issue because COX-2 may play important roles in both the ER and the Golgi. COX-2 in the ER could serve to augment COX-1-mediated PG formation at relatively higher cytosolic Ca\(^{2+}\) concentrations when cPLA\(_2\) is translocated to the ER as well as the Golgi (46, 70). COX-2 in the ER and nuclear envelope also could be involved in generating another PG product(s) (e.g., PGL\(_2\)). Further studies are needed to examine the relationship between cell activation in, for example, immune cells and COX-2 trafficking to the Golgi.

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