The 19-amino Acid Cassette of Cyclooxygenase-2 Mediates Entry of the Protein into the Endoplasmic Reticulum-associated Degradation System

Received for publication, August 30, 2006 · Published, JBC Papers in Press, September 25, 2006, DOI 10.1074/jbc.M608281200

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Cyclooxygenase (COX) isoenzymes catalyze the committed step in prostaglandin biosynthesis. The primary structures of COX-1 and COX-2 are very similar except that COX-2 has a 19-amino acid (19-AA) segment of unknown function located just inside its C terminus. Here we provide evidence that the major role of the 19-AA cassette is to mediate entry of COX-2 into the ER-associated degradation system that transports ER proteins to the cytoplasm. COX-1 is constitutively expressed in many cells, whereas COX-2 is usually expressed inducibly and transiently. In murine NIH/3T3 fibroblasts, we find that COX-2 protein is degraded with a half-life ($t_{1/2}$) of about 2 h, whereas COX-1 is reasonably stable ($t_{1/2} > 12$ h); COX-2 degradation is retarded by 26 S proteasome inhibitors. Similarly, COX-1 expressed heterologously in HEK293 cells is quite stable ($t_{1/2} > 24$ h), whereas COX-2 expressed heterologously is degraded with a $t_{1/2}$ of $\sim 5$ h, and its degradation is slowed by proteasome inhibitors. A deletion mutant of COX-2 was prepared lacking 18 residues of the 19-AA cassette. This mutant retains native COX-2 activity but, unlike native COX-2, is stable in HEK293 cells. Conversely, inserting the COX-2 19-AA cassette near the C terminus of COX-1 yields a mutant $ins^{594–612}$ COX-1 that is unstable ($t_{1/2} \sim 3$ h). Mutation of Asn-594, an N-glycosylation site at the beginning of the 19-AA cassette, stabilizes both COX-2 and $ins^{594–612}$ COX-1; nonetheless, COX mutants that are glycosylated at Asn-594 but lack the remainder of the 19-amino acid cassette (i.e. $del^{597–612}$ COX-2 and $ins^{594–596}$ COX-1) are stable. Thus, although glycosylation of Asn-594 is necessary for COX-2 degradation, at least part of the remainder of the 19-AA insert is also required. Finally, kifunensine, a mannosidase inhibitor that can block entry of ER proteins into the ER-associated degradation system, retards COX-2 degradation.

Prostanoids are an important class of lipid mediators that are synthesized in almost all mammalian tissues. Prostanoids act in an autocrine and paracrine fashion through G protein-coupled receptors to elicit a variety of physiological and pathological responses (1–7). The committed step in the prostaglandin biosynthetic pathway is catalyzed by the cyclooxygenase isozymes COX-1 $^2$ and COX-2 (1, 3, 4, 8). Within a species, COX-1 and COX-2 exhibit $\sim 60$% amino acid sequence identity. Both isoforms are membrane-bound, ER-resident, heme-containing glycoproteins that function as homodimers (1, 3, 4, 9). Structural and biochemical studies have shown that COX-1 and COX-2 are unusual integral membrane proteins in that the enzymes associate monotypically with the luminal surfaces of the ER and the contiguous inner membrane of the nuclear envelope (10–15).

Despite their close similarities in structure, catalytic function, and subcellular localization, COX-1 and COX-2 differ markedly in their profiles of expression. COX-1 is constitutively expressed in resting cells of many tissues (16). COX-2 expression can be induced in fibroblast, epithelial, endothelial, macrophage, and smooth muscle cells in response to growth factors, cytokines, and proinflammatory stimuli and expression is usually transient (reviewed in Refs. 1, 17, and 18). Differences in the patterns of expression of COX-1 and COX-2 genes have been quite clearly delineated in serum-stimulated NIH/3T3 fibroblasts (19–21). COX-1 mRNA and protein levels remain unchanged before and after serum stimulation of quiescent NIH/3T3 cells. In contrast, COX-2 mRNA and protein are barely detectable prior to stimulation, become significantly up-regulated shortly after stimulation, and then rapidly decline to basal levels. It is well established that the different profiles of COX-1 and COX-2 expression as observed in serum-stimulated NIH/3T3 fibroblasts are partly attributable to differences in regulation of the isoforms at the transcriptional and post-transcriptional levels (1, 17, 18). It is not clear the degree to which COX-1 and COX-2 may also be regulated differently at the post-translational level. However, rapid degradation of COX-2 protein may serve a significant physiological role in regulating the levels of prostanoids whose syntheses occur via this isoform. Previous studies have indicated that COX-2 can be ubiquitinated and degraded by the 26 S proteasome in the cytoplasm (22–24), suggesting that COX-2 degradation can involve

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§ This work was supported by National Institutes of Health Grant GM68848. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ The abbreviations used are: COX, cyclooxygenase; KIF, kifunensine; 19-AA insert, the unique C-terminal 19-amino acid insert of COX-2; ER, endoplasmic reticulum; ERAD, ER-associated degradation; LPS, lipopolysaccharide; CHX, cycloheximide; Endo H, endoglycosidase H; ov, ovine; hu, human; mu, murine; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

$^2$ The abbreviations used are: COX, cyclooxygenase; KIF, kifunensine; 19-AA insert, the unique C-terminal 19-amino acid insert of COX-2; ER, endoplasmic reticulum; ERAD, ER-associated degradation; LPS, lipopolysaccharide; CHX, cycloheximide; Endo H, endoglycosidase H; ov, ovine; hu, human; mu, murine; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline.
exit from the ER via the ER-associated degradation (ERAD) system(s) followed by proteolysis by the proteasome (25–32).

In the present study, we establish that COX-2 protein is preferentially and rapidly degraded in NIH/3T3 and HEK293 cells under conditions in which COX-1 is very stable. We also confirm that although COX-2 is an ER-resident enzyme, its degradation is proteasome-dependent. In investigating the molecular basis for the different rates of COX-1 and COX-2 degradation, we found that the 19-amino acid cassette unique to COX-2 (Asn-594–Lys-612) and located 6 residues in from the C-terminal end targets the protein for entry into the ERAD pathway(s) (29, 33, 34).

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, pronase E, tetracycline, and goat serum were from Invitrogen. Bovine calf serum was from Hyclone. Cycloheximide, puromycin, and bacterial lipopolysaccharide (LPS) were obtained from Sigma. Kifunensine, MG132, epoxomicin, E64, and leupeptin were purchased from Calbiochem. Endoglycosidase H (Endo H) was purchased from Roche Applied Science. Cold arachidonic acid was purchased from Cayman Chemicals, whereas [1-14C]arachidonic acid (55 mCi/mm01) and [1-14C]eicosapentaenoic acid (55 mCi/mm01) were from American Radiolabeled Chemicals.

**Construction of Plasmids for Transfection**—Recombinant ovine (ov) COX-1 cDNA and human (hu) COX-2 cDNA were subcloned into pLND (Invitrogen) and pcDNA5/FRT/TO (Invitrogen), respectively. pLND is edcsyone-inducible, whereas pcDNA5/FRT/TO is tetracycline-inducible. After subcloning, the QuikChange™ site-directed mutagenesis kit (Stratagene) was used to create the following C-terminal mutants: N594A huCOX-2, del595–612 huCOX-2, del 597–612 huCOX-2, and ins594–596 ovCOX-1. The COX-1 insertion mutants, ins594–612 ovCOX-1 and ins594–612(N594A) ovCOX-1, were created from the cDNA template for native ovCOX-1 by overlap extension PCR and then subcloned into pLND using HindIII restriction sites. Correct cDNA orientation and mutations were confirmed by sequencing.

**Cell Culture and Transfection**—NIH/3T3 fibroblasts at early passage (<6 passages) were cultured in DMEM supplemented with 10% bovine calf serum and 100 units/ml penicillin/streptomycin. To induce COX-2 expression, the cells were first made quiescent by serum starvation for 48 h in DMEM containing 0.2% bovine calf serum and thereafter treated with DMEM supplemented with 20% FBS for 4 h.

RAW 264.7 macrophage-like cells were cultured in DMEM supplemented with 10% FBS and 100 units/ml penicillin/streptomycin. To stimulate COX-2 expression, the cells were challenged with 200 ng/ml LPS.

HEK293-derived cell lines stably expressing native or mutant COX constructs were generated using either the tetracycline-inducible or the edcsyone-inducible mammalian expression systems (Invitrogen) according to the manufacturer’s protocol. Constructs that were expressed under the control of a tetracycline-inducible promoter were native huCOX-2, del595–612 huCOX-2, N594A huCOX-2, and del 597–612 huCOX-2; those expressed under the control of the edcsyone-inducible promoter were native ovCOX-1, ins594–612 ovCOX-1, ins594–612(N594A) ovCOX-1, and ins594–596 ovCOX-1. Stably transfected HEK293 cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin/streptomycin, and the appropriate pharmacological selective reagents. Inducible expression was achieved by a 24-h serum starvation in serum-free medium followed by treatment with 10 µg/ml tetracycline or 10 µM Kifunensine (KIF) analog for the appropriate times in normal culture medium.

**Protein Degradation and Drug Treatments**—A cycloheximide (CHX) decay experiment was performed to measure the protein stabilities of COX-1 and COX-2 in serum-treated NIH/3T3 cells. Briefly, quiescent cells were serum-stimulated for 4 h and then incubated for different times with 50 µM CHX in DMEM supplemented with 10% FBS. The protein degradation experiment was performed in the absence or presence of MG132 (20 and 50 µM), epoxomicin (3 and 5 µM), 25 µM leupeptin, and 25 µM E64.

HEK293 cells inducibly expressing wild-type or mutant cyclooxygenase constructs were grown to 80% confluency, serum-starved for 24 h, and then treated with 10 µg/ml tetracycline or 10 µM KIF in complete culture medium to induce expression. Afterward, the cells were incubated for various times with 50 µM puromycin to block translation in the absence or presence of 20 µM MG132, 25 µM Kifunensine (KIF), or 50 µM KIF.

**Enzymatic Deglycosylation**—For complete deglycosylation of cell proteins, HEK293 whole cell lysates were denatured by boiling in NuPAGE SDS sample loading buffer (Invitrogen) and then treated for at least 12 h with Endo H at a concentration of 0.4 milliunits/µl.

**Western Transfer Blotting**—After the appropriate treatments, NIH/3T3, RAW 264.7, and HEK293 cells were scraped into ice-cold PBS (phosphate-buffered saline), pH 7.4, containing 5 mM EDTA and a mixture of protease inhibitors (Roche Applied Science) and lysed by sonication. Radioimmune precipitation lysis buffer (10 mM Tris, pH 7.2, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate) containing a protease inhibitor mixture (Roche Applied Science) was also used for cell lysis. Protein concentrations were determined using the BCA protein assay kit (Pierce). The NuPAGE system (Invitrogen) was used to resolve the proteins in the whole cell lysates on a 7% Tris-acetate polyacrylamide gel. After transfer to a nitrocellulose membrane, immunoblotting was performed with the appropriate primary antibody. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (Bio-Rad) were used as secondary antibodies. Immunodetection was performed using the Western Lighting chemiluminescence kit (Amersham Biosciences) followed by exposure to x-ray film. Densitometry analysis was performed using ImageQuant TL software (Amersham Biosciences).

**Antibodies Specific for COX-1 or COX-2**—A previously generated (12), peptide-specific, polyclonal primary antibody for murine (mu) COX-2 against the epitope Ser-598–Lys-612 was used in the current study to detect muCOX-2 by immunoblotting. A polyclonal antibody raised against whole recombinant ovCOX-1 was used to detect muCOX-1. Peptide-specific, polyclonal primary antibodies for ovCOX-1 and huCOX-2 were
Cyclooxygenase Degradation

A.  

| Time with CHX (h) | COX-2 | COX-1 | Actin |
|------------------|-------|-------|-------|
| 0                |       |       |       |
| 1                |       |       |       |
| 2                |       |       |       |
| 4                |       |       |       |
| 6                |       |       |       |
| 12               |       |       |       |

B.  

| % Protein Remaining | COX-1 | COX-2 |
|---------------------|-------|-------|
| Time after CHX (h)  |       |       |
| 0                   | 100   | 100   |
| 2                   | 90    | 90    |
| 4                   | 80    | 80    |
| 6                   | 70    | 70    |
| 8                   | 60    | 60    |

C.  

| Time with CHX (h) | No protease inhibitor | 5 µM Epox | 25 µM Leup |
|------------------|----------------------|----------|---------|
| 0                | 100                  | 100      | 100     |
| 2                | 90                   | 90       | 90      |
| 4                | 80                   | 80       | 80      |
| 6                | 70                   | 70       | 70      |
| 8                | 60                   | 60       | 60      |

D.  

| % Protein Remaining | CHX only | CHX + MG132 |
|---------------------|----------|-------------|
| Time with CHX (h)   |          |            |
| 0                   | 100      | 100         |
| 2                   | 90       | 90          |
| 4                   | 80       | 80          |
| 6                   | 70       | 70          |
| 8                   | 60       | 60          |

FIGURE 1. Cyclooxygenase protein degradation in serum-treated NIH/3T3 cells. A, quiescent 3T3 cells were stimulated with 20% FBS for 4 h to induce COX-2 expression. CHX (50 µM final concentration) was added to the medium, and the cells were incubated for the indicated times. Cell lysates were then prepared and analyzed by Western blotting for COX-1, COX-2, and actin as described under “Experimental Procedures.” B, densitometry was performed to quantitate the levels of COX-1 and COX-2, which were normalized to those of actin. Error bars denote ± S.E. of the mean for three independent experiments. C, COX-2 expression was induced in murine NIH/3T3 cells, and 50 µM CHX added to the medium as described in A. The cells were then treated for the indicated times with or without 3 or 5 µM epoxomicin (Epox), 25 µM leupeptin (Leup), or 50 µM MG132, COX-2 protein levels were examined by Western blotting and densitometry. D, quantitative analysis of the effect of 20 µM MG132 on COX-2 protein stability in 3T3 cells. Densitometry values for each time point were from six independent experiments performed as described above and represent the mean ± S.E. Asterisks denote that a p value < 0.05 based on a Student’s paired t test.
Cyclooxygenase Degradation

Rapid, Proteasome-dependent Degradation of COX-2—COX-1 is expressed constitutively in murine NIH/3T3 fibroblasts, whereas COX-2 is expressed inducibly and transiently (19–21). This raises the possibility that the protein stabilities of the COX isoforms are different with COX-2 being more susceptible to degradation. A protein decay experiment was performed with serum-stimulated, CHX-treated NIH/3T3 cells to compare the stabilities of COX-1 and COX-2 (Fig. 1, A and B). COX-2 was degraded with a short half-life (t\(1/2\)) of \(\sim 2\) h, whereas COX-1 was more stable and did not appear to undergo degradation during the experiment (t\(1/2\) > 12 h).

Although COX-2 expression is typically transient, a few tissues and cell types are reported to express COX-2 constitutively (22, 37–40). It is possible that COX-2 protein is stable under these conditions. To address this, we examined COX-2 protein degradation in LPS-stimulated murine RAW264.7 macrophage cells where COX-2 protein expression continues to increase for up to 24 h after initiating LPS treatment (41, 42). COX-2 was also degraded rapidly (t\(1/2\) \sim 2 h) in LPS-challenged RAW 264.7 cells (data not shown), indicating that increased COX-2 protein stability is not responsible for prolonged expression of the enzyme at least in this cell type.

We performed experiments with several classes of protease inhibitors to determine the pathway responsible for the selective degradation of COX-2 in NIH/3T3 cells. The 26 S proteasome inhibitors epoxomicin and MG132 (Fig. 1, C and D) significantly slowed COX-2 protein degradation at 4 and 8 h after CHX treatment, whereas two different cysteine protease inhibitors of lysosomal degradation, leupeptin (Fig. 1C) and E64 (data not shown), did not. At the 2-h time point in six independent experiments with 20 \(\mu\)M MG132, there was a trend but not a statistically significant inhibition (Fig. 1D).

**RESULTS**

| A. | Asn68 | Asn144 | Asn410 | RPPTEL COOH |
|----|-------|--------|--------|-------------|
| Signal | EGF | MBD | Globular -Catalytic | COX-1 |

| B. | 585FSVPDPEL1KT1VT1NASSDRSLDDINPTVLLK358 |
|----|--------------------------------------|
| 585FSVPDPEL1KT1VT1NASSDRSLDDINPTVLLK358 | native huCOX-2 |
| 585FSVPDPEL1KT1VT1NASSDRSLDDINPTVLLK358 | del595–612 huCOX-2 |
| 585FSVPDPEL1KT1VT1NASSDRSLDDINPTVLLK358 | N594A huCOX-2 |
| 585FSVPDPEL1KT1VT1NASSDRSLDDINPTVLLK358 | del597–612 huCOX-2 |
| 585FSVPDPEL1KT1VT1NASSDRSLDDINPTVLLK358 | ins594–612 ovCOX-1 |
| 585FSVPDPEL1KT1VT1NASSDRSLDDINPTVLLK358 | ins594–612(N594) ovCOX-1 |
| 585FSVPDPEL1KT1VT1NASSDRSLDDINPTVLLK358 | ins594–596 ovCOX-1 |

| C. | native ovCOX-1 | native huCOX-2 |
|----|----------------|----------------|
| Actin | 0 | 4 | 8 | 12 | 24 | h |
| t\(1/2\) > 24 h | t\(1/2\) ~ 5 h |
| del595–612 huCOX-2 | ins594–612 ovCOX-1 |
| Actin | 0 | 4 | 8 | 12 | 24 | h |
| t\(1/2\) > 24 h | t\(1/2\) ~ 3 h |

| D. | native huCOX-2 |
|----|----------------|
| No protease inhibitor | 20 \(\mu\)M MG132 |
| 0 | 4 | 8 | 12 | 24 | h |
| ins594–612 ovCOX-1 |
| 0 | 4 | 8 | 12 | 24 | h |

FIGURE 2. Stability of heterologously expressed native and C-terminal cyclooxygenase mutants in HEK293 cells. A, alignment of major folding domains of COX-1 and COX-2 showing consensus N-glycosylation sites and the relative position of the unique 19-AA insert located near the C terminus of COX-2. Numbering for COX-1 begins with the Met at the translation start site. Numbering for COX-2 parallels the COX-1 numbering in which the start of the mature, processed COX-2 protein has the same number as the start of the mature, processed COX-1 (11). EGF, epidermal growth factor; MBD, membrane binding domain. B, amino acid sequences of the C termini of cyclooxygenase constructs that were stably transfected into HEK293 cells. The underlined sequence is the consensus N-glycosylation site at the start of the unique COX-2 19-AA cassette.

Proteins that were expressed under the control of a tetracycline-inducible promoter were native ovCOX-1, del595–612 ovCOX-2, N594A ovCOX-2, and del597–612 ovCOX-2; those expressed under the control of the ecdysone-inducible promoter were ins594–612 ovCOX-1, ins594–612(N594A) ovCOX-1, and ins594–596 ovCOX-1. C, HEK293 cell lines stably and inducibly expressing the constructs shown in B were grown to ~80% confluency, subjected to serum starvation for 24 h, and then treated with the appropriate inducing agent (20 \(\mu\)g/ml tetracycline or 10 \(\mu\)M ponasterone A) for 24 h (native huCOX-2, del595–612 huCOX-2, N594A huCOX-2, and del597–612 huCOX-2; two different cysteine protease inhibitors of lysosomal degradation, leupeptin (Fig. 1E) and E64 (data not shown), did not. At the 2-h time point in six independent experiments with 20 \(\mu\)M MG132, there was a trend but not a statistically significant inhibition (Fig. 1D).

viously described (36). Briefly, purified enzyme (~23 milliunits) was incubated with a 100-\(\mu\)l reaction mixture containing 0.1 M Tris-HCl, pH 8.0, 1 mM phenol, 10 \(\mu\)M hematin, and [1-\(14\)C]arachidonic acid or [1-\(14\)C]eicosapentaenoic acid at room temperature for 30 s. The reaction was terminated by adding 300 \(\mu\)l of an ice-cold mixture of ethylether, methanol, 0.2 M citric acid (30:4:1). The organic phase of the reaction mixture, containing the radioactive products, was isolated and applied directly onto a TLC silica plate at 4 °C. The plate was devel-
Cyclooxygenase Degradation

Accordingly, it is not clear whether a protease other than the 26 S proteasome can also be involved in COX-2 degradation.

It should be noted that both 26 S proteasome inhibitors prevented the degradation of the two alternatively glycosylated forms (i.e. 72 and 74 kDa) of COX-2 (Fig. 1, C and D). Overall, our findings and those of others (22–24) indicate that COX-2 can be degraded in the cytosol by the 26 S proteasome. Because COX-2 is located on the luminal surface of the ER (12–15), our results imply that its degradation must involve transport across the ER membrane to the cytosol.

The Unique C-terminal 19-amino Acid Segment of COX-2 Is Involved in COX-2 Degradation—Despite the high degree of structural similarity between the COX isoforms, COX-2 is distinct from COX-1 in possessing a unique 19-AA cassette that is located 6 residues from the C terminus (Fig. 2A). The N-terminal-most residue of the 19-AA insert is Asn-594, which is one of four functional N-glycosylation sites of COX-2 (43) (44); COX-1 has the other three N-glycosylation sites in common with COX-2. We used site-directed mutagenesis to test the hypothesis that the unique 19-AA segment of COX-2 affects its degradation. HEK293 cells, which do not have detectable levels of COX-1 or COX-2, were used to stably and inducibly express native or C-terminal mutant versions of the cyclooxygenase enzymes (Fig. 2B). Native human (hu) COX-2 inducibly expressed in HEK293 cells was degraded with a \( t_{1/2} \) of \( \sim 5 \) h, whereas native ovine (ov) COX-1 was not degraded at a detectable rate \( (t_{1/2} > 24) \) (Fig. 2C). A deletion mutant del595–612 huCOX-2 was prepared that lacks 18 amino acids of the 19-AA cassette segment; additionally, Asn-594 cannot be glycosylated in this mutant as the consensus N-glycosylation sequence is no longer present. This deletion mutant was stable with a \( t_{1/2} \) comparable with that of native ovCOX-1 (\( > 24 \) h) (Fig. 2C). Inserting the 19-AA cassette of huCOX-2 near the C terminus of ovCOX-1 yielded a mutant ins594–612 ovCOX-1 that had a relatively short \( t_{1/2} \) of \( \sim 3 \) h (Fig. 2C). Degradation of native COX-2 and of ins594–612 ovCOX-1 was retarded by treatment with the proteasome inhibitor MG132 (Fig. 2D). These observations suggest that the 19-AA segment of COX-2 is involved in the rapid degradation of this COX isoform, perhaps by targeting it for proteolysis by the 26 S proteasome.

The native and mutant proteins used in the studies depicted in Fig. 2 and in subsequent figures are inducibly expressed in HEK293 cells with different time courses. To the extent possible, we performed protein degradation experiments with HEK cells expressing comparable amounts of COX protein to achieve a more direct comparison of protein half-lives.

To compare the properties of native and mutant versions of COX-2, we determined the \( K_m \) and \( V_{max} \) values of native murine (mu) COX-2 and del595–612 muCOX-2. We also analyzed the products formed from \([1^{-14}C] \)arachidonic acid and \([1^{-14}C] \)eicosapentaenoic acid by both enzymes. The kinetic constants and product distributions were the same for the native and mutant enzymes (Fig. 3, A and B). Additionally, we determined the subcellular distribution of the native and mutant proteins heterologously expressed in HEK293 cells (Fig. 4). Both mutant enzymes exhibited a perinuclear and diffuse cytoplasmic staining pattern characteristic of COX-1 and COX-2 (12, 13, 45); we also determined by Western blotting that del595–612 huCOX-2 and ins594–612 ovCOX-1 are present in a high speed, microsomal membrane fraction prepared from HEK293 cells (data not shown). Collectively, our results suggest that effects on substrate turnover or subcellular localization do not account for the large difference in the rates of degradation between native COX-2 and del595–612 COX-2.

Asn-594 Glycosylation Is Necessary but Not Sufficient to Effect COX Degradation—A point mutuation of the Asn-594 N-glycosylation site at the beginning of the 19-AA segment was sufficient to extend the \( t_{1/2} \) of the mutated COX-2 (NS594A huCOX-2) to \( > 24 \) h (Fig. 5A). Consistent with this, ins594–612 ovCOX-1 was stabilized by mutating the Asn-594 glycosylation site (Fig. 5A). However, a deletion mutant del597–612 huCOX-2 carrying the Asn-594 consensus N-glycosylation site but lacking the rest of the 19-AA insert was as stable as del595–612 huCOX-2 (Fig. 5B). Similarly, insertion of a consensus N-glycosylation site near the C ter-

![FIGURE 3. Kinetic properties of purified native muCOX-2 and del595–612 muCOX-2. A, hexahistidine-tagged versions of muCOX-2 and del595–612 muCOX-2 were expressed in Sf21 insect cells and purified as described under “Experimental Procedures.” Cyclooxygenase assays were performed as described under “Experimental Procedures.” B, similar amounts of purified hexahistidine-tagged muCOX-2 or del595–612 muCOX-2 (~23 milliunits) were incubated with 20 \( \mu \)M \([1^{-14}C] \)arachidonic acid (AA) or \([1^{-14}C] \)eicosapentaenoic acid (EPA) for 30 s. The products were extracted, separated by thin-layer chromatography, and visualized by autoradiography as described under “Experimental Procedures.” The radioactive band between EPA and PGH3 has chromatographic properties of the PGH3 degradation product 17-hydroxy-5,12,15-heptadecatrienoic acid.](image-url)
minus of ovCOX-1 (ins\textsubscript{594–596} ovCOX-1) did not destabilize this COX isoform (Fig. 5B), although this mutant is at least partly glycosylated at Asn-594 (Fig. 6A, panel I).

In evaluating the glycosylation status of Asn-594 in various forms of COX-1 and COX-2, we compared the molecular mass of ins\textsubscript{594–612} ovCOX-1 with that of ins\textsubscript{594–612(N594A)} ovCOX-1 and the mass of native huCOX-2 to that of N594A huCOX-2. The mass of an N-linked oligosaccharide moiety is \(1 \text{ kDa} \) (43). Both ins\textsubscript{594–612} ovCOX-1 and native huCOX-2 would be expected to have a total of four N-glycosylation sites (43, 44). As anticipated, the electrophoretic mobility of ins\textsubscript{594–612} ovCOX-1 was less than that of ins\textsubscript{594–612(N594A)} ovCOX-1 by a difference corresponding to about 2 kDa (Fig. 6A, panel I); moreover, deglycosylation of ins\textsubscript{594–612} ovCOX-1 and ins\textsubscript{594–612(N594A)} ovCOX-1 by Endo H increased the mobilities of both mutants such that they had apparently identical molecular masses (Fig. 6A, panel II). These results suggest that the difference in molecular mass between the two COX-1 insertion mutants results from glycosylation of ins\textsubscript{594–612} ovCOX-1 at Asn-594.

In contrast to the results obtained with the COX-1 mutants, we found no difference between the electrophoretic mobilities of native huCOX-2 and N594A huCOX-2 expressed in HEK293 cells (Fig. 6B, panel I). This is somewhat similar to what is seen with murine COX-2 expressed in \(cos\)-1 cells (43) where the major bands for native muCOX-2 and N594Q muCOX-2 have the same electrophoretic mobilities; however, in the case of the muCOX-2 expressed in \(cos\)-1 cells, a more slowly moving band was also observed. A second, less mobile band was observed with huCOX-2, but not N594A huCOX-2, when HEK cells were treated with KIF, an \(\alpha_2\) ER mannosidase I inhibitor (Fig.

**FIGURE 4.** Subcellular localization of native huCOX-2, del\textsubscript{595–612} huCOX-2, native ovCOX-1, and ins\textsubscript{594–612} ovCOX-1 in HEK293 cells. HEK293 cells stably expressing the various COX variants were grown on poly-L-lysine-coated coverslips and induced with either 10 \(\mu\)g/ml tetracycline or 10 \(\mu\)M ponasterone A as described in the legend for Fig. 2. The cells were then fixed with formaldehyde, permeabilized with Triton X-100, and incubated with an appropriate primary antibody. A, for huCOX-2 (panel I) and del\textsubscript{595–612} huCOX-2 (panel II), a rabbit anti-peptide antibody to residues 583–594 of huCOX-2 was used. B, for native ovCOX-1 (panels I and II) and ins\textsubscript{594–612} ovCOX-1 (panel III), a rabbit anti-peptide antibody to residues 272–283 of ovCOX-1 was used for panel I, whereas an antibody against whole ovCOX-1 was used for panels II and III. After washing with PBS, the cells were incubated with fluorophore-conjugated anti-rabbit IgG secondary antibody, washed with PBS, mounted using an anti-fade reagent, and examined by fluorescence microscopy at a magnification of \(x200\).

**FIGURE 5.** Stability of cyclooxygenase mutants in HEK293 cells having various modifications of the 19-amino acid cassette. A and B, amino acid sequences of the C termini of cyclooxygenase constructs that were stably transfected in HEK293 cells are shown in Fig. 2B. Proteins that were expressed under the control of the tetracycline-inducible promoter were N594A huCOX-2 and del\textsubscript{597–612} huCOX-2; those expressed under the control of the ecdysone-inducible promoter were ins\textsubscript{594–612(N594A)} ovCOX-1 and ins\textsubscript{594–612} ovCOX-1. Expression was induced for 12 h (N594A huCOX-2 and del\textsubscript{597–612} huCOX-2) or 24 h (ins\textsubscript{594–612(N594A)} ovCOX-1 and ins\textsubscript{594–612} ovCOX-1). Thereafter, the time course of degradation of the proteins was determined as described in the legend for Fig. 2. The results shown in A are representative of at least three independent experiments from which protein half-life measurements were made. The results shown in B are representative of two independent experiments.
Cyclooxygenase Degradation

A. (I) 1 2 3 4
1. native ovCOX-1
2. ins594-596 ovCOX-1
3. ins594-612 ovCOX-1
4. ins594-612 (N594A) ovCOX-1

B. (I) 1 2
1. native huCOX-2
2. N594A huCOX-2

(II) 1 2 3
1. ins594-612 ovCOX-1
2. ins594-612 (N594A) ovCOX-1
3. Endo H cleavage of 1
4. Endo H cleavage of 2

(II) native huCOX-2

1. tet-induced
2. tet-induced + 12 h KIF
3. Endo H cleavage of 1
4. Endo H cleavage of 2

C. Actin

0 2 4 8 24

Time with puromycin (h)

2 4 8 24

25 μM KIF

6B, panel II). Moreover, Endo H treatment led to deglycosylated forms of native COX-2 and N594A COX-2 that had the same molecular mass (Fig. 6B, panel II).

In addition to causing the appearance of a second, alternatively glycosylated form of huCOX-2, KIF treatment of HEK293 cells expressing native huCOX-2 inhibits COX-2 degradation (Fig. 6B, panel II). Degradation of ins594 – 612 ovCOX-1 is also inhibited by KIF (Fig. 6C). This is a diagnostic feature of N-glycosylated proteins whose degradation involves transport to the ERAD system. These results are consistent with those obtained with 26 S proteasome inhibitors in indicating that COX-2 degradation involves transport to the ERAD system prior to degradation by the 26 S proteasome.

DISCUSSION

Substitution of the COX-1 coding region for COX-2 in transgenic mice partially rescues the typical phenotype of COX-2 null mice.3 Thus, a key difference between COX-1 and COX-2 is in their patterns of expression and not simply in their enzymatic properties. The difference in the profiles of COX-1 and COX-2 protein expression is at least partly attributable to differences in the regulation of transcription of the two genes (reviewed in Refs. 1, 17, and 18). In general, COX-1 gene expression is constitutive, and COX-2 expression is inducible. In addition, COX-2 mRNA with its repetitive AUUUA degradation sequences is subject to post-transcriptional control (52–54). In this report, we confirm and extend previous findings indicating that COX-1 is a stable protein, whereas COX-2 is degraded relatively rapidly, and that COX-2 can be ubiquitinated and degraded by the 26 S proteasome (22–24). COX-2 is localized to the luminal surface of the ER (12–15), and so to be degraded, it must first be transported to the cytosol from the ER.

In the studies reported here, we have focused on the molecular basis for the relatively rapid rate of COX-2 degradation when compared with that of COX-1. Specifically, we evaluated the 19-AA cassette (Asn-594–Lys-612) that is unique to COX-2 for its potential role in degradation. This 19-AA insert is located 6 residues in from the COX-2 C terminus. It has only one recognizable consensus sequence, an N-glycosylation sequence involving Asn-594–Ser-596.

We have demonstrated that the 19-AA cassette of COX-2, with an intact Asn-594 N-glycosylation site, targets COX-2 for proteasomal degradation. This is based on the following observations. a) A deletion mutant of huCOX-2 lacking 18 amino acids of the 19-AA insert (del595–612 huCOX-2) and a functional N-glycosylation site at Asn-594 has native COX activity and the same subcellular location as native enzyme but is refractory to protein degradation. b) Point mutation of the Asn-594 N-glycosylation site (N594A huCOX-2) also stabilizes COX-2. c) Inserting the COX-2 19-AA cassette at the C terminus of ovCOX-1 (ins594–612 ovCOX-1) disrupts ovCOX-1. d) Point mutation of Asn-594 in ins594–612 ovCOX-1 stabilizes this insertion mutant. e) The degradation of both native huCOX-2 and ins594–612 ovCOX-1 can be retarded by MG132, a 26 S proteasome inhibitor. Although the Asn-594 N-glycosylation site is required for degradation, we found evidence that all or part of the remainder of the 19-AA insert is also necessary.

Our results suggest that glycosylation of Asn-594 of COX-2 is critical for initiating entry of the enzyme into the ERAD sys-

3 C. Funk, personal communication.
system for transport to the cytoplasm. The ERAD system is thought to involve unfolding of N-glycosylated proteins in the ER lumen and ATP-dependent retrograde transport across the ER membrane, and frequently, this is followed by ubiquitination of the transported protein by ubiquitin-protein isopeptide (E3) ligases associated with the cytosolic face of the ER membrane and subsequent degradation by the 26 S proteasome (25–32). The ERAD system is usually regarded as a quality control pathway for the clearance of misfolded, unassembled, or toxic proteins from the ER lumen (25–29). It has also been implicated in the degradation of native ER-associated proteins such as hydroxymethylglutaryl-CoA reductase, hepatic microsomal cytochrome P450 CYP3A4, and inositol 1,4,5-triphosphate receptor (55–57), all of which are ER transmembrane proteins. An ER luminal glycoprotein is selected for the ERAD pathway by a complex process that involves enzymatic processing of specific N-linked oligosaccharide groups on the surface of glycoproteins (25, 33, 58–60). This requires the action of α,1,2 ER mannosidase I whose inhibition by KIF leads to retention and stabilization of glycoproteins in the ER (46–51). In the current study, we show that KIF retards the degradation of COX-2 and a COX-1 mutant containing the 19- AA cassette of COX-2.

Spear and Ng (59) have recently shown that in a multiply glycosylated glycoprotein a single, specific N-linked oligosaccharide group serves as the determinant for entry into the ERAD pathway. In the present study, we have provided evidence suggesting that only one of the four N-glycosylation sites of COX-2 (i.e. Asn-594) is involved in COX-2 degradation; however, degradation also requires at least some amino acids downstream of the consensus N-glycosylation sequence. We presume that a carbohydrate moiety linked to Asn-594 along with adjacent amino acids of the 19-A A insert interact with the complex of proteins that provide entry into the ERAD system. Very little of the 19- AA cassette can be resolved in the COX-2 crystal structure (11), and therefore, the 19- AA insert is thought to lack secondary structure. The last resolved residue in the murine COX-2 crystal structure is Ser-596, the final amino acid in the N-glycosylation consensus sequence. Ser-596 is solvent-exposed and appears to be oriented in such a way that its C terminus would point toward the lipid bilayer. The COX-2 KDEL-like ER retention motif at the C terminus (45) also has to be on the protein surface to be recognized (61). Thus, the 19- AA cassette of COX-2 is likely to be solvent-exposed and situated close to the membrane surface.

In summary, we have identified a 19- AA cassette having a consensus N-glycosylation site at its N terminus that can target a protein to the ERAD system. Scanning mutagenesis should be helpful in identifying the critical residues in the 19- AA cassette that work in conjunction with the Asn-594 N-glycosylation site to target COX-2 for degradation. It will also be important to characterize the oligosaccharide group linked to Asn-594.

Acknowledgments—We thank Dr. Anne Vojtek and Jennifer Taylor for the kind help with immunocytofluorescence. We are also grateful to Dr. Billy Tsai for the thoughtful suggestions relevant to this work.

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