This is to certify that the
dissertation entitled

ROLE OF C-TERMINAL 18 AMINO ACIDS FOR
THE BIOLOGIGAL ACTIVITY OF
PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE-2

presented by

Hui-yuan Tang

has been accepted towards fulfillment
of the requirements for the

Ph.D degree in Biochemistry Molecular Biology

Major Professor's Signature

12/7/07

Date

ROLE OF C-TERMINAL 18 AMINO ACIDS FOR THE BIOLOGIGAL ACTIVITY OF PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE-2

By

Hui-yuan Tang

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry and Molecular Biology

2007

ABSTRACT

ROLE OF C-TERMINAL 18 AMINO ACIDS FOR THE BIOLOGICAL ACTIVITY OF PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE-2

By

Hui-yuan Tang

Cyclooxygenases-1 and -2 are membrane-localized heme-containing homodimers that catalyze the committed step in prostaglandin synthesis. COX-2 protein has a unique 18-amino acid cassette located four residues from the carboxyl terminus of all COX-2 species that is not found in COX-1. The hypothesis that I investigated was that this 18-amino acid cassette is responsible in part for the distinct biological activity of COX-2 by mediating protein-protein interactions. 293 T-Rex cell lines that inducibly express Flag-tagged native COX-2, COX-2 *del*581-598 (mutant with amino acids 581-598 deleted), COX-1 and COX-1 *ins*580-598 (mutant with amino acids 580-598 of COX-2 inserted near the amino terminal), or 293 Freestyle cells that transiently express Flag-tagged native COX-2 were used to identify potential protein partners for the cyclooxygenase isoforms. Two proteins were identified by proteomic analyses that reproducibly co-purified with Flag-tagged COX-2, FAM44A (GI/Q8NFC6) and Heat shock-induced protein (GI/188492). FAM44A has been reported as a 330KDa protein with an AT-hook DNA binding domains and FAM44A protein can be phosphorylated upon DNA damage. The interaction between COX-2 and FAM44A could be an alternative way to regulate cell cycle progression. Heat shock proteins, like Heat shock-induced protein usually act like chaperones to guide protein folding. Thus, the Heat shock-induced protein we identified may help process COX-2 or be involved in its degradation. No reproducible protein partners were identified for COX-2 *del*581-598 or COX-1. Interestingly, the

FAM44A protein was also purified with COX-1 *ins*580-598, which contains the 18-amino acid insert, providing further evidence that it may interact specifically with the 18-amino acid cassette of COX-2. One role of the 18-amino acid cassette is to mediate the degradation of COX-2 via the ER-associated degradation (ERAD) system. Kifnusenin, an inhibitor of terminal fucosidases that increases the COX-2 stability, was applied to 293 T-Rex cells expressing Flag-tagged COX-2 in an attempt to trap protein partners of COX-2 that might be involved in protein degradation. No additional proteins were identified with this treatment. Yet other COX-2 protein partners may exist whose interactions are too transient or have too low affinity to survive purification. Untransfected 293 cells do not express endogenous COX-2, and may not also express other protein partners for COX-2. The role of the 18-amino acid cassette on cell biology was also investigated. Our results demonstrated that 18-amino acid insert had no effect on cyclooxygenase activity, but reduced the number of colonies that could be detected that stably expressed COX-2. This may be due to reduce protein stability of COX-2 compared to COX-2 *del*581-598. The 18 amino acid insert had no effect on COX-2 protein subcellular location or cell growth.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor Dr. David L. DeWitt for his wonderful tutoring throughout my Ph.D study. His incredible guidance, technically and intellectually, has helped me accomplish this challenging thesis project. Without his continuous encouragement, I wouldn't have been able to successfully complete my graduate career. Also I would like to make a grateful acknowledgement for my committee members. Their helpful advice and innovative ideas were extremely useful in my research.

I am appreciative for all of the lab members, present or past. The friendship, technical help, and career advice they offered has helped me through my graduate study. I would like to thank Robin Goodwin for all the kind support I received ever since I joined the lab. I have constantly turned to her for suggestions on many issues and have learned a lot from her. I would like to thank Christi Hemming for giving me useful advice on both work and life. She has been a good friend that I can rely on and I enjoyed working with her. I would like to thank Doug Whitten for teaching me how to analyze the Mass Spectrometry data and all the helpful discussion. I would like to thank Drs. Uri R. Mbonye, Jiayan Liu and Christine Harman for all the thought-provoking discussion and advice. Thank you all for providing me such a pleasant environment to practice my research study.

Finally I would like to thank my family for their selfless support and love. It is impossible for me to achieve anything without them.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (APPENDIX)

LIST OF FIGURES

LIST OF FIGURES (APPENDIX)

Images in this thesis/dissertation are presented in color.

ABBREVIATIONS

18 aa	C-terminal 18-amino acid cassette of COX-2
3'-UTR	3' untranslated region
AA	arachidonic acid
ADP	adenosine diphosphate
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATP	ATM and Rad3 related
ARE	AU-rich motif
CHX	cycloheximide
COX	cyclooxygenase
CREB	cAMP regulatory binding protein
EGF	epidermal growth factor
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FBS	fetal bovine serum
FLAP	5-lipoxygenase activating protein
HEME	ferric iron protoporphyrin IX
HMG	non-histone chromosomal protein (high mobility group)
Hu	human
iNOS	inductible nitric oxide synthase
KIF	kifunensine
MBD	membrane binding domain
mPGES	microsomal prostaglandin E synthase
NSAID	non-steroidal anti-inflammatory drug
Ov	ovine
PBS	phosphate buffered saline
PEI	polyethylenimine
PGHS	prostaglandin endoperoxide H synthase
PG	prostaglandin
POX	peroxidase
PPAR	peroxisome proliferator-activated receptors
TEV	tobacco etch virus
Tet	tetracycline
$T_{1/2}$	half-life

CHAPTER I

LITERATURE REVIEW

Introduction

Prostaglandin endoperoxide H synthase-1 and 2 (PGHS-1 and PGHS-2; also cyclooxygenase-1 and 2, COX-1 and COX-2) are membrane-localized heme-containing homodimers that localize to the luminal side of the endoplasmic reticulum (ER) membrane and to the inner and outer nuclear membranes (1). They catalyze the committed steps in prostaglandin synthesis: a cyclooxygenase reaction in which arachidonate plus two molecules of O_2 are converted to Prostaglandin G_2 (PGG_2) and a two electron peroxidase reduction of PGG_2 to PGH_2 (Fig. 1). Various prostaglandin synthases then catalyze the isomerization or reduction of PGH_2 to the biological active prostaglandins such as prostaglandin E_2 (PGE_2), prostaglandin F_2 (PGF_2), prostacyclin (PGI_2) (Fig. 1).

Prostaglandins are unsaturated carboxylic acids, consisting of a 20-carbon skeleton with a five member ring. Prostaglandins are ubiquitous autocrine/paracrine regulators that mediate a wide range of physiological functions, such as control of cell growth, contraction of smooth muscle, and modulation of inflammation (2-4).. Prostaglandin receptors are a subfamily of cell surface seven-transmembrane G-protein-coupled receptors, and are classified into 9 subgroups: DP1-2, EP1-4, FP, IP, and TP (5), that respond to the prostaglandins D, E, $F_{2\alpha}$, prostacyclin and thromboxane, respectively. Subtypes of PG-specific receptors couple with different signaling pathways. For example, the 4 PGE receptor subtypes individually regulate intracellular Ca^{2+} mobilization, as cAMP cyclase activity, and phosphodiesterase

activity (6, 7). Thus the same prostaglandin can have opposing biological functions, depending on which subtype is expressed in a cell or the concentration of PG product.

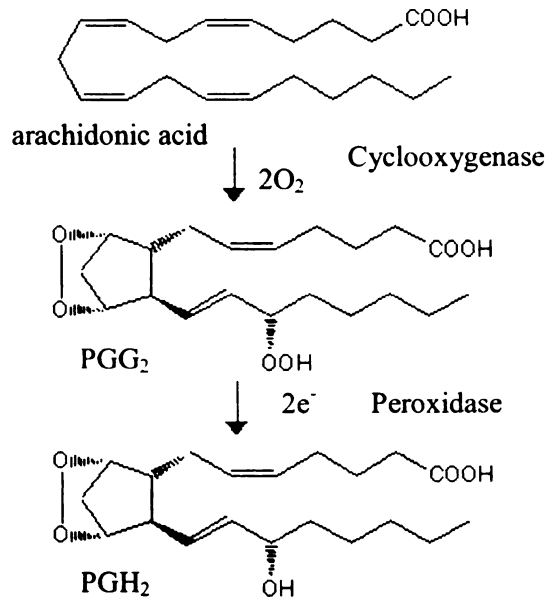


Figure 1. Biosynthetic pathway for the formation of prostanoids derived from arachidonic acid. Phospholipid is cleaved by the phospholipase A_2 to generate the arachidonic acid. The COX enzymes convert arachidonic acid to form PGH_2 by two steps. Various terminal synthases then convert PGH_2 to different prostanoids.

The primary structures of COX-1 and -2 from many species are known (1) (Fig 2). COX-1 and 2 contain different lengths of signal peptides that are cotranslationally cleaved from the nascent polypeptide by microsomal signal peptidases. At the COX-1 and -2 carboxyl termini is a four amino acid sequence STEL, thought to be an analogue to the KDEL retention sequence necessary for retention of proteins in the endoplasmic reticulum (8). Mature COX-1 and COX-2 contain 576 and 587 amino

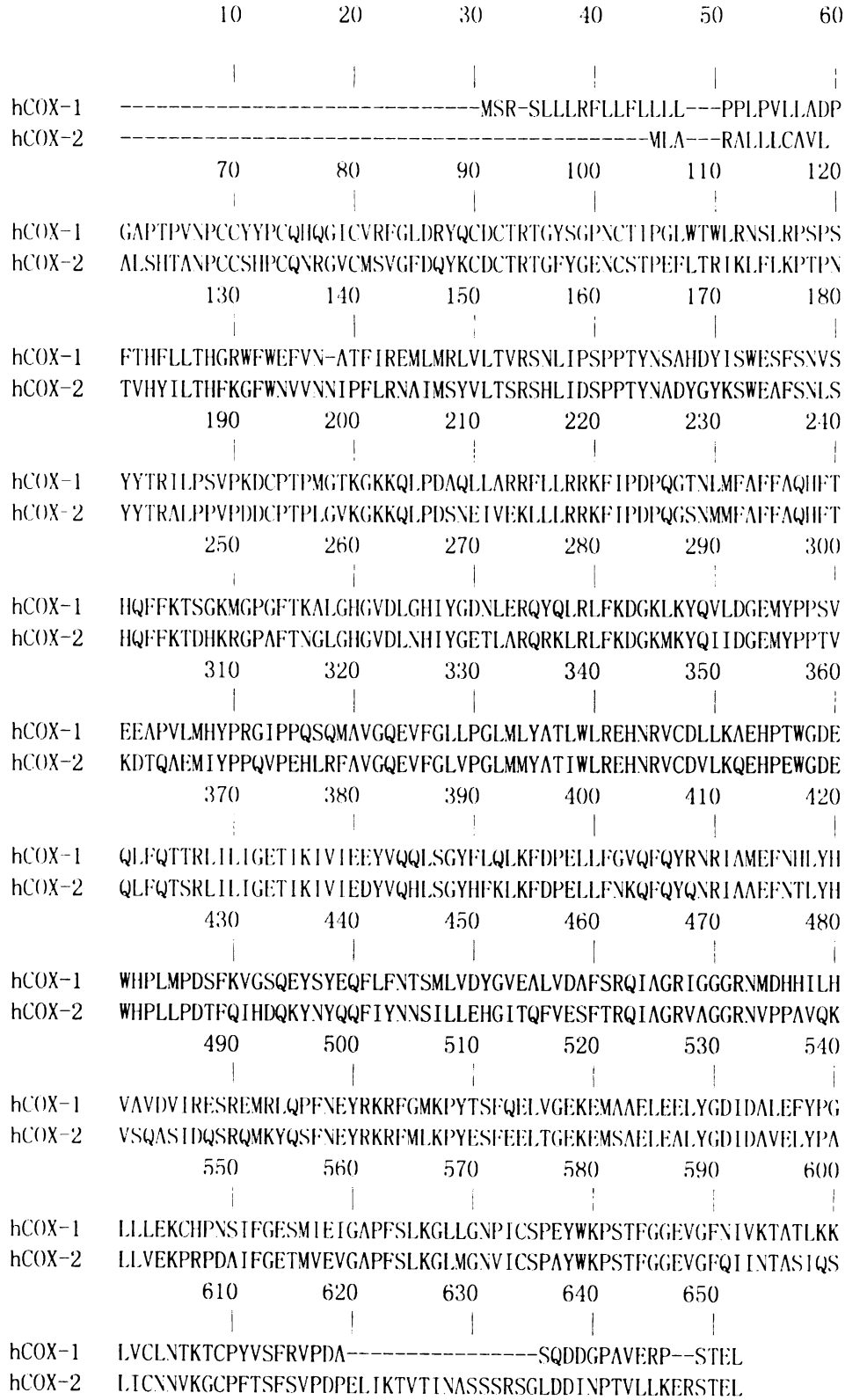


Figure 2. Sequence alignment of Human COX-1 and -2

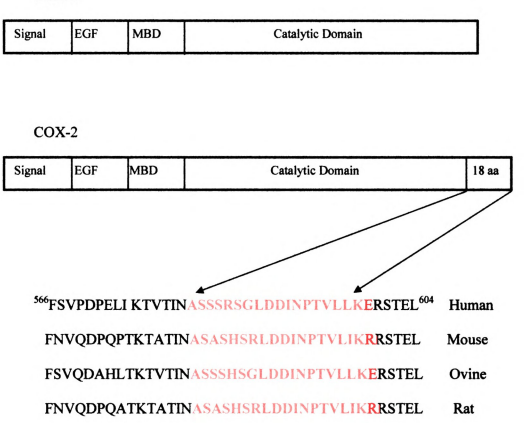


Figure 3. Domain Structures of COX-1 and COX-2. Both COX isoforms have N-terminal signal peptides, EGF-like domains, membrane binding domains and catalytic domains. The most significant difference in primary structure between the two is the presence of a unique 18-residue cassette near the C-terminal end of COX-2.

acids and share 60%–65% amino acid sequence homology (9). The major sequence differences between COX isoforms occur in the membrane binding domains and at the carboxyl termini, where a unique 18-amino acid sequence is present at the C terminus of COX-2 and not in COX-1. The function of this 18-amino acid is the topic of these studies (Fig. 3).

COX-1 and COX-2 are encoded by separate genes, *Ptgs-1* and *Ptgs-2*, respectively. While the proteins share similar structures and kinetic properties, COX-1 and COX-2 have different expression pattern and are involved in different physiological processes. COX-1 is constitutively expressed in most tissues and is thought to produce prostaglandins that serve housekeeping functions (10, 11). COX-2, however, is not expressed in most unstimulated tissues but is rapidly induced by growth factors, cytokines, and tumor promoters (12, 13). Prostaglandins produced by COX-2 regulate inflammation, differentiation, mitogenesis and angiogenesis (14).

Reaction Mechanism of Cyclooxygenase

The reaction mechanism of cyclooxygenase is shown in Fig 4. The peroxidase activity is required for cyclooxygenase activity; however peroxidase activity itself is independent. For the first step of the reaction, peroxide reacts with the heme group. Then a two-electron oxidation occurs, forming compound I and alcohol (15-18) (Fig. 4). Compound I can be rearranged by a single electron oxidation of Tyr^{385} to form intermediate II which contain an oxyferryl group (Fe(IV)=O), a neutral protoporphyrin IX, and a Tyr^{385} tyrosyl radical which is required for cyclooxygenase activity (15, 16, 19, 20). As mentioned before, compound I can continue to cycle through the peroxidase reaction independently from cyclooxygenase turnover (21).

When the cyclooxygenase site is occupied by a fatty acid substrate, intermediate II can abstract the hydrogen atom from C_{13} in arachidonate to form an radical (22) (Fig. 1 and 4). The fatty acid radical then reacts with molecular O_2 to produce an 11-hydroperoxyl radical which in turn cyclizes to form a C_{11}-C_9

endoperoxide bridge. A second O_2 molecule adds at C_{15} to produce PGG_2. PGG_2 is reduced to PGH_2 by the peroxidase activity of COX.

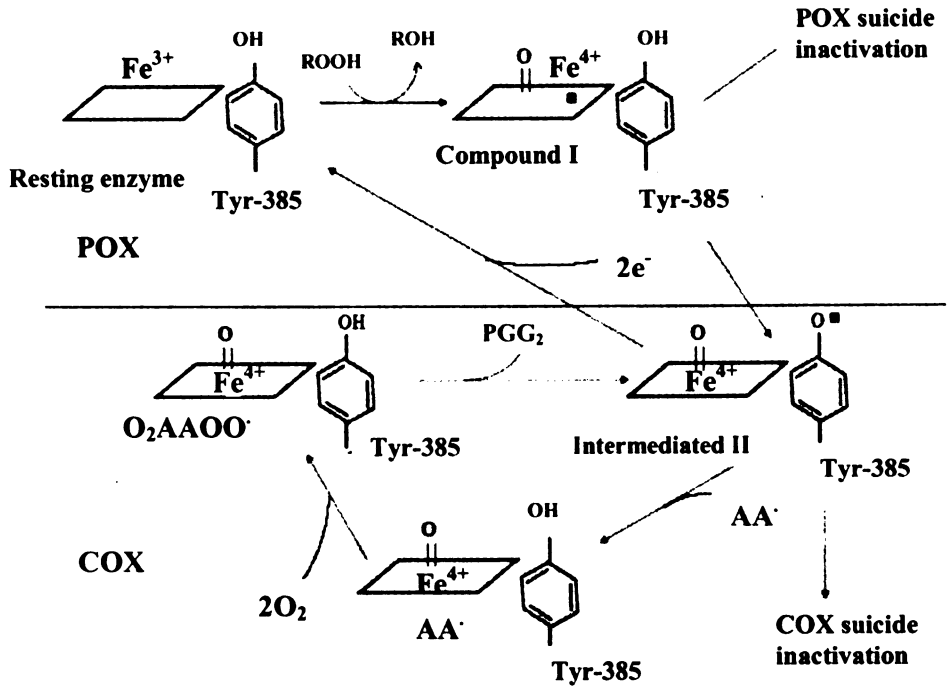


Figure 4. Cyclooxygenase and peroxidase catalysis and suicide inactivation of COXs. Fe^{3+} PPIX, ferric iron protoporphyrin IX (heme); ROOH, alkyl hydroperoxide; ROH, alcohol; AA, arachidonic acid; Fe^{4+}=PPIX, oxyferryl heme. Compound I can form intermediate II or alternatively undergo a one electron reduction by an exogenous electron donor, yielding compound II. Intermediate III is a spectral intermediate of unknown structure but perhaps involving a heme group with a protein radical located on an amino acid side chain other than Tyr385.

One interesting point is that both the cyclooxygenase or peroxidase activity of COX-1 and -2 rapidly catalytically inactivate in the presence of sufficient substrate. One probable mechanism for this suicide inactivation is shown in Figure 4. It involves the production of a Tyrosine^{385} radical (intermediate II) (23) and migration of the

radical on Tyr^{385} to another tyrosine within the protein that leads to protein damage by side chain cross linking or some other poorly understood mechanism. Consistent with this idea are findings that protein tyrosyl radicals in oPGHS-1 are observed on tyrosines other than Tyr^{385} (24–26), and that an intermediate III has been detected whose time course of production parallels peroxidase inactivation (23). It should be mentioned that the rates of both peroxidase and cyclooxygenase inactivation can be noticeably slowed by peroxidase-reducing cosubstrates (23, 27, 28). Reducing cosubstrates may increase the efficiency of conversion of intermediate II to compound II, and reduce production of intermediate III. Suicide inactivation is an interesting chemical phenomenon whose biological relevance is unclear.

Crystal Structures of Cyclooxygenase

Crystal structures of COX-1 and COX-2 have been determined by the Garavito and Luong laboratories (29, 30) (Fig. 5) and are very similar. In his initial report on the structure of COX-1, Garavito defined separate and distinct protein domains involved in dimerization, membrane binding and catalysis (29). The C-terminal tails are not visible in the crystal structures, presumably because of their flexibility. COX-1 and COX-2 dimers are held together via hydrophobic interactions, hydrogen bonding, and salt bridges between the dimerization domains of each monomer. Three disulfide bonds hold the EGF domain together. The catalysis domain of COX enzymes includes a cycloxygenase and a peroxidase active site. For the two-step conversion of arachidonate to prostaglandin H_2, the first step takes place in the cyclooxygenase active site to form the peroxide intermediate, prostaglandin G2. PGG_2 then next diffuses out of the cyclooxygenase site to the peroxidase active site on the opposite

side of the protein, where it is reduced to prostaglandin H_2. The COX enzymes have an unusual and unique mechanism of interaction with the membrane (Fig. 5).

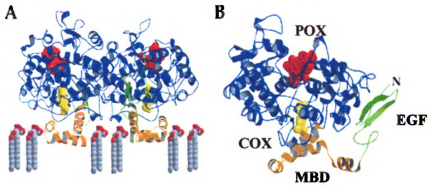


Figure 5. Structure of ovine prostaglandin endoperoxidase H synthase-1 (oCOX-1) (Picot et al., 1994). (*A*) oCOX-1 homodimer associates with the luminal face of the ER membrane. Three major folding domains are: epidermal growth factor domain for dimerization (EGF; *green*), membrane binding domain (MBD; *gold*) and globular catalytic domain (*blue*) which contains peroxidase (*red*) and cyclooxygenase (*yellow*) active sites. (*B*) The oCOX-1 monomer with the locations of the peroxidase (POX) and cyclooxygenase (COX) active sites and the EGF and membrane binding (MBD) domains. The color scheme is the same as in (*A*).

Although these enzymes are integral membrane proteins, they do not have any transmembrane sequences; instead four amphipathic helices form a hydrophobic surface that anchors these enzymes on the membrane. These amphipathic helices not only form the base of the molecule but they also form the entrance to the cyclooxygenase active site, which is a hydrophobic pocket that projects inward from the membrane surface of the enzyme. These helices interact at the membrane solvent interface. It is likely that fatty acid substrates and NSAIDs (Non-steroidal anti-

inflammatory drugs) also partition to this interfaced region where they are accessible to the cyclooxygenase active site (30).

Cyclooxygenase Inhibitors

Inhibitors of cyclooxygenase belong to the class of drugs referred to as non-steroidal anti-inflammatory drugs (NSAIDs). All NSAIDs inhibit arachidonate binding to the cyclooxygenase active site of COX-1 and 2. Non selective NSAIDs inhibit both COX-1 and -2, while most selective inhibitor preferentially inhibits COX-2. NSAIDs use can have adverse side effects including peptic ulceration and dyspepsia. These side effects are believed to be due to the inhibition of prostaglandin synthesis by COX-1, which produces prostaglandins that mediate protective reaction in the gastrointestinal mucosa. Drugs that selectively inhibit COX-2 have reduced gastrointestinal toxicities. Celecoxib and rofecoxib are the two COX-2 selective NSAIDs. To understand the mechanism for nonselective and selective inhibition of COX enzymes, it is necessary to examine how arachidonate and nonselective NSAIDs bind within the active sites of COX-1 and COX-2. Arg^{120} is one of the few charged amino acids at the cyclooxygenase active site of COX. Crystal structure indicates that the guanidinium group of Arg^{120} can form a salt bond with the carboxylic moiety of arachidonate (31). The carboxyl of arachidonate anchored near the mouth of the hydrophobic pocket and the hydrophobic tail of arachidonate insert into the hydrophobic pocket, forming a hairpin turn between carbons 9 and 11 (Fig. 6). This orientation allows for the addition of two molecules of oxygen at carbon 9, 11 and 15 resulting in the formation of prostaglandin G_2. Evidence from mutagenesis has also shown that Arg^{120} is required for COX-1 activity and for the binding and inhibition by

acidic cyclooxygenase inhibitors, which constitute the largest group of nonselective NSAIDs (32) (Fig. 7). Interestingly, nonacidic (COX-2 selective) NSAIDs, such as Dup697 and L-746, inhibit the R120E COX-1 mutant about 10 times more efficiently than the native COX-1, suggesting that Arg^{120} may actually discourage binding of

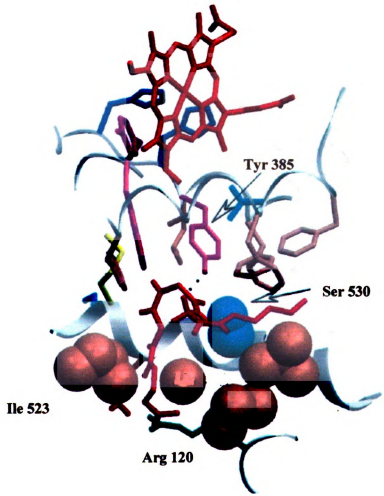


Figure 6. Hypothetical orientation of arachidonate within the substrate binding pocket of ovine COX-1 (R. Kurumbail, Second International Workshop on COX-2, 1998). Binding of arachidonate within the COX-1 active site is dependent on coordination with Arg^{120} (green).

nonacidic inhibitors in COX-1 (33). In contrast to its function in COX-1, Arg^{120} plays only an accessory role for NSAIDs and fatty acid binding in COX-2 (33, 34). The COX-2 isozyme also has a larger cyclooxygenase pocket than COX-1 (30). This increased size allows fatty acids and inhibitors to more readily to access to the COX-2 active site, which diminishes the relative importance of ionic interactions with Arg^{120}. Another effect of the larger COX-2 active site is that steric crowding causing by Arg^{120} at the entrance of the pocket may be reduced and thereby increases the access

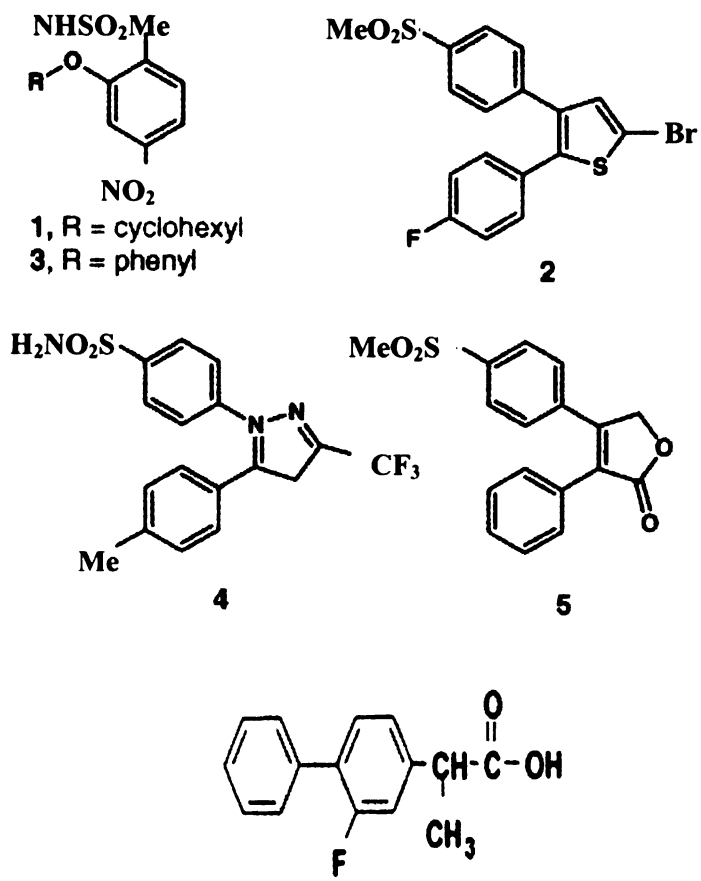


Figure 7. Structure of representative nonselective and COX-2 selective NSAIDs NS-398(1), DuP 697(2), nimesulide(3), celecoxib(4), rofecoxib(5), Flurbiprofen (6).

of nonacidic inhibitors in COX-2. Therefore Arg^{120} may play an indirect role in the drug selectivity by discriminating against binding of nonacidic NSAIDs more effectively in the COX-1 binding site than in the larger COX-2 binding site.

Besides the Arg^{120} residue, several other amino acids in cyclooxygenase binding site influence the volume and chemical environment of the COX-2 active site, and contribute to the selective inhibition. The subsituition of $Valine^{523}$ in COX-2 with an Isoleucine in COX-1 (35, 36) produced a much larger binding site in COX-2 than in COX-1 (Fig. 8) because of the shorten side chain of Valine. This amino acid contacts directly with the inhibitors. Substitution of $Phenlyalanine^{503}$ with Leucine in COX-2 has added effect of allowing to a larger binding pocket, filled by most COX-2 inhibitor (36, 37).

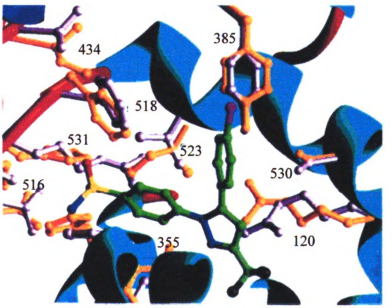


Figure 8. Superposition of COX-1 (yellow) to COX-2 (purple) around SC-558 (Kurumbail et al., 1996). The larger binding pocket is COX-2 is clearly visible.

Regulation of Cyclooxygenase Gene Expression

Transcription Regulation

Because COX-1 is expressed constitutively in most tissues, and expression levels of this enzyme do not vary greatly in adult animals, it has been difficult to study transcriptional regulation of the COX-1 gene. The COX-1 gene has a TATA-less promoter that contains multiple start sites for transcription (38). Gel shift assays have demonstrated that Sp1 *cis*-regulatory elements in the human COX-1 promoter, at positions -111/-105 and-610/-604, bind the *trans*-activating Sp1 protein (39). Deletion of either site leads to a reduction of about 50% in basal transcription, and deletion of both sites results in a reduction of about 75% (38). To date these Sp1 sites are the only *cis*-acting elements documented to regulate transcription of COX-1.

Although numerous possible regulatory elements have been identified in the COX-2 genes, only five have been rigorously demonstrated to regulate transcription: overlapping E-box and ATF/CRE sequences near the TATA box, an NF/IL6 CAAT enhancer binding sequence upstream, and two NFκB binding sites at -214 and 427 (Fig. 10). The most critical of these regulatory sequences is the ATF/CRE, a regulatory element that typically is activated by hetero- and homodimers of the c-Fos, c-Jun, and ATF families of bZIP proteins (AP-1) (40), and the cAMP regulatory binding protein (CREB). The ATF/CRE and NF/IL6 regulatory elements will also cooperate to activate the COX-2 expression in human epithelial cells (41). The E-box is required for hormonal regulation of COX-2 in rat granulosa cells (42) and for the elevated expression of COX-2 in the murine carcinoma cell line JWF2 (43). NFκB signaling has been implicated variously in the expression of COX-2 stimulated by TNFα, hypoxia, endothelin, and IL-1β in osteoblastic cells (12), synoviacytes (44, 45),

epithelial cells (46, 47), endothelial cells (48), and hepatocytes (49). Each of these effectors, as well as LPS, can activate the NFκB signaling pathway (50, 51).

Another important signal pathway: the MAPK pathways have been shown to contribute to the increased expression of COX-2 in one or more cultured cell systems in response to inflammatory stimuli, including IL-1β, TNFα, and LPS, as well as the phorbol ester TPA (52, 53). Dependence on kinase signaling for COX-2 expression has been demonstrated by overexpressing active kinases, or conversely by using dominant negative mutant kinases (54-56), and by using small molecule inhibitors that selectively block one or more of the ERK1/2, JNK/SAPK, and p38/RK/Mpk2 pathways (56-65).

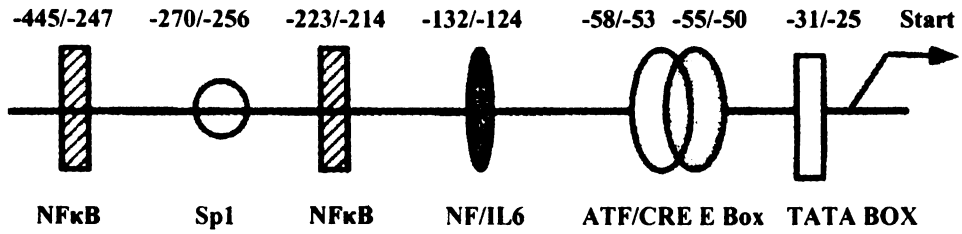


Figure 9. Regulatory elements in the human COX-2 promoter (Yamamoto, 1995)

Post-transcriptional Regulation

COX-2 mRNA has a very short half life due to the presence of multiple copies of the AUUUA motif within the 3'-UTR of COX-2 mRNA that are known to regulate mRNA stability (66, 67). Deletion of these motifs in COX-2 stabilizes the transcript (66, 68). COX-1 mRNA lacks these AU-rich motif (AREs) and is very stable (68). The mechanism by which AREs promote mRNA degradation in mammalian cells is not clearly understood. There is general agreement that AREs promote deadenylation

of the polyA tail which precedes 5' to 3' or 3' to 5' exonuclease cleavage of the mRNA (69-72).

The ARE binding protein(s) responsible for initiating ARE-mediated decay of COX-2 mRNA are yet to be identified. However many proteins have been found that can bind to the COX-2 3'-UTR and stabilize the transcript. For example, HuR-mediated COX-2 transcript stabilization has been reported in colon cancer cells where COX-2 is aberrantly over-expressed (73).

Cyclooxygenase protein turnover

Recent data from the Smith's laboratory has indicated that degradation of COX-2 occurs via the ER-associated degradation (ERAD) system in 293 cells (74). Kifnusenin, an inhibitor of terminal fucosidases stabilizes the protein (74). Consistent with this idea, Rockwell et al., has observed the accumulation of COX-2 in its native form and as polyubiquitin conjugates in HT4 neuronal-like cells treated with inhibitors of proteasomal degradation, while COX-1 protein levels were unchanged by this treatment (75). These experimental results suggest that COX-2 may be selectively regulated by the ubiquitin-proteasome pathway, which has been implicated in the degradation of intracellular proteins with short half-lives. Rapid turnover of COX-2 in tissues where the enzyme is transiently expressed may serve a significant physiological role in regulating the levels of prostanoids whose synthesis is attributed to this COX isoform.

Different Biological Activity of Cyclooxygenases

COX-1 and COX-2 are involved in different physiological processes. Mouse knock out studies clearly show primary roles for COX-1 in platelet aggregation (76) and parturition (77, 78), and for COX-2 in ovulation, implantation (79), and neonatal development (80, 81). COX-2 knock out mice have multiple defects in ovulation, implantation, and decidualization (82); in contrast COX-1 knock-out mice have lengthened bleeding time and difficulty with parturition (83). To date, most studies have focused the role of COX-2 in carcinogenesis, less well studied are the role of COX-1 (84, 85). Increased COX-2 expression is sufficient to cause formation of breast tumors in transgenic mice (84). Non-steroidal anti-inflammatory drugs (NSAIDS) reduce carcinogen-induced mammary tumors in rats (85). In $Apc^{\Delta 761}$ knockout mice, a model for inherited colorectal cancer (FAP), genetic disruption of COX-2, or NSAIDS that selectively inhibit COX-2, suppress polyp formation (86). These studies confirm an obligate role for COX-2 overexpression in the transformation process. It is not clear how COX-2 facilitates transformation. COX-2's roles in oncogenesis have variously been attributed to the stimulatory effects of prostaglandins on cell growth (87-89), to inhibition of apoptosis (90), and to activation of the nuclear PPAR receptors (91-93). However, deficiency in either COX-1 or COX-2 reduces polyp formation in $Min^{-/+}$ mice almost equally (94, 95). So an unanswered question is whether these two contribute to carcinogenesis via the same or different mechanisms. One plausible explanation for the COX isozymes roles in colon cancer is that COX-1 provides prostaglandins that protect carcinogen initiated stem cells from DNA damage-induced apoptosis, whereas COX-2 promotes transformation after loss of heterozygosity of the Apc gene.

There has been no good explanation for the apparent redundancy of COX-1 and 2. These two enzymes are so similar that it is difficult to explain why their biological activities are different. Difference in regulation, protein turnover, and the subtle catalytic differences towards arachidonic acid favors COX-2 at low substrate levels may be important, but other factors may also be involved. My thesis will attempt to investigate the biochemical mechanism for the unique biological activity of COX-2.

CHAPTER II

PUTATIVE PROTEIN PARTNERS FOR CYCLOOXYGENASE PROTEINS

Summary

There has been no good explanation for the apparent redundancy of COX-1 and 2. These two enzymes are so similar that it is difficult to explain why their biological activities are different. The COX-2 protein has a unique 18-amino acid cassette located four residues from the carboxyl terminus of all COX-2 species that is not found in COX-1. Experiments in our lab have demonstrated that deletion of this 18 amino acid sequence does not affect catalytic activity. The hypothesis that I investigated was that this 18-amino acid cassette is responsible in part for the distinct biological activity of COX-2 by mediating protein-protein interactions. Two proteins were identified by proteomic analyses that reproducibly co-purified with Flag-tagged COX-2 in 293 cells: FAM44A (GI/Q8NFC6) and Heat shock-induced protein (GI/188492). FAM44A was identified in 5 out of the 7 experiments in protein complexes with COX-2 purified from T-Rex 293 cells, and 1 in 2 experiments where COX-2 was expressed transiently in Freestyle 293 cells. FAM44A protein was also identified once with COX-1 *ins*580-598, which provided further evidence for its specific interaction with the COX-2 18-amino acid cassette. FAM44A is a 330kDa protein that has been identified previously from cDNA data. FAM44A can be phosphoylated during DNA damage. The interaction between COX-2 and FAM44A could be involved in DNA damage induced cell cycle checkpoint control. The heat shock-induced protein was only identified in 1 out of the 7 experiments in protein complexes with COX-2 purified from T-Rex 293 cells, and 1 in 2 experiments where

COX-2 was expressed transiently in Freestyle 293 cells. It may function as a chaperone in the process of COX-2 or be involved in its degradation. Kifnusenin, an inhibitor of terminal fucosidases which increases the stability of COX-2, was applied to 293 T-Rex cells expressing Flag-tagged COX-2 in an attempt to trap protein partners of COX-2 that might be involved in protein degradation. No new unique proteins were identified even with this treatment. No noteworthy protein partners were identified for COX-2 *del*581-598 or COX-1. Other COX-2 protein partners may exist that were missed due to the fact that their interactions are transient, or have low affinity and do not survive purification. Furthermore untransfected 293 cells do not express endogenous COX-2, and may not also express the normal protein partners for COX-2.

Introduction

That protein-protein interactions may modify the activity of COX-2 is a speculative hypothesis, but there is circumstantial evidence to support its validity. The position of the 18 amino acid insert near the carboxyl terminal would be ideal to allow interactions with other proteins. The carboxyl terminus of COX-2 cannot be observed in crystal structures; usually an indication of regions that are very flexible, suggesting that the COX-2 tail would be free to interact with other proteins. In addition, cytokine-stimulated prostaglandin synthesis proceeds selectively via newly expressed COX-2 in many cell systems, even when COX-1 is present. Such synthesis is often channeled through a coordinately-expressed membrane associated PGE_2 synthase (96-99). The mechanism whereby COX-2 selectively provides PGH_2 to prostaglandin synthases may be via protein-protein interactions of COX-2 with a channel protein. 5-

lipoxygenase, the enzyme that catalyzes the initial step in leukotriene biosynthesis requires the 5-lipoxygenase activating protein (FLAP) for the arachidonate substrate (100, 101). Nuclear receptors, PPARs, may also mediate the biological effects of COX-2-derived prostaglandins by direct regulation of gene expression by prostanoids (91-93). Nevertheless, such a mechanism requires the nuclear transport of prostaglandins. Since no cyclooxygenase is present in the nucleus, accessory proteins would be needed to import prostaglandins.

Nitric oxide produced by inducible NO synthase (iNOS) and prostaglandins generated by COX-2 have been reported to be involved in inflammation (102, 103). Since the inducible expression of the two enzymes by inflammatory stimuli has the similar time course, suggests that the two systems may interact. Recently direct interaction of iNOS and COX-2 has been revealed (104). Endogenous COX-2 and iNOS can be induced by LPS in RAW264.7 cells and co-immunoprecipitation confirmed their interaction. GST pull down assay carried out in HEK293 cells that transiently co-expressing iNOS and COX-2 also proved the direct binding between these two proteins. Mutagenesis showed that the binding occurred at the C-terminal domain of COX-2 which was in accordance with our hypothesis. The interaction seems to bring NO in proximity to COX-2, S-nitrosylate COX-2 and enhance the catalytic activity of COX-2. The molecular synergism between iNOS and COX-2 may represent a major mechanism of inflammatory responses. However iNOS is soluble and found predominantly in the cytosol while COX-2 is localized to the ER and nuclear membrane. In order to further confirm the interaction, their subcellular localization should be examined to verify that they can localize together.

It has been demonstrated before that COX-2 may be degraded by the ubiquitin proteasome system (UPS) (74, 75). However, how ubiquitination is accomplished and

regulated was unclear. An important regulator of the UPS is the COP9 signalosome (CSN), which controls the stability of many proteins. Recent data in Dubiel's lab have confirmed that COX-2 can physically interact with the CSN using density gradient centrifugation and immunoprecipitation (105). Pull down experiments with Flag-tagged COX-2 revealed that COX-2 was associated with large complexes consisting of the CSN, cullin-RING Ub ligases and the 26S proteasome which further proved the proteasome-dependent degradation of COX-2 in HeLa cell lysate.

Our understanding of the different biological roles of COX-1 and COX-2 is only beginning to emerge. The functions of these two isozymes in apoptosis, particularly as it relates to the development of a variety of cancers (e.g. colon, lung, and breast), angiogenesis, respiration, inflammation, pain, and reproduction are currently being studied in cultured cells and whole animals. If specific protein interactions were identified, this would provide a more complete explanation for COX-2 signal transduction and its role in transformation. In addition, a better understanding of signaling through the COX pathway could also lead to more specific chemoprevention for a variety of cancers.

Experimental Procedures

Materials. FreeStyle™ 293 cell line and Flp-In T-Rex 293 cell line were from Invitrogen. Dulbecco's modified Eagle medium (DMEM), Lipofectamine 2000, cellfectin, tetracycline, blasticidin, zeocin, hygromycin, and penicillin-streptomycin were from Invitrogen/Gibco. Fetal bovine serum (FBS) was purchased from Atlas Biologicals. Cycloheximide and puromycin were obtained from Sigma-Aldrich. Kifnusenin was purchased from Calbiochem.

Construction of the Flag-tagged COX-2 and COX-2 *del*581-598 in the pFastBac vector for Infection. FLAG (DYKDDDDK) and TEV (CENLYFQG) sequences were inserted into the N-terminus of human (h) COX-2 cloned into the pFastBac vector (Appendix, Fig. 22). Flag-tagged COX-2-pFastbac was used as a template to make the deletion mutant (ASSSRSGLDDINPTVLLK) by PCR mediated mutation. The Flag tag binds to the anti-Flag peptide, which when coupled to agarose beads, can be used for the affinity purification; TEV (Tobacco Etch Virus) sites are recognized and cleaved by the TEV protease which allows protease-mediated elution from the Flag affinity column. The plasmids were used to produce baculoviruses, which were used to infect Sf21 insect cells. High levels of expression in insect cells allowed us to confirm that if the deletion and the insertion of the tag affected hCOX-2 activity. The primers, vector and PCR conditions used to design the above mutants are shown in the 'Appendix' section (Table 7).

Construction of Flag-tagged COX-1 and COX-1 *ins*580-598 in the pIND vector. The histidine-tagged ovine (o) COX-1 and COX-1 *ins*580-598 subcloned into the pIND vector at *Hind III* site were a gift from the laboratory of Dr. William Smith. FLAG (DYKDDDDK) sites were inserted by PCR amplification into the N-terminus of ovine COX-1 to replace the His tag (HHHHHH) in the pIND vector using specific primers (Appendix, Table 7) (Appendix, Fig. 23).

Construction of pCDNA expression plasmids for the Flag-tagged COX-2, COX-2 *del*581-598, COX-2 N580A, COX-2 Y371F, COX-1 and COX-1 *ins*580-598. Flag-tagged COX-2 and COX-2 *del*581-598 were subcloned from pFastbac into a pcDNA5/FRT/TO vector (Appendix, Fig. 24). COX-1 and COX-1 *ins*580-598 were also subcloned from pIND into a pcDNA5/FRT/TO vector (Appendix, Table 8). N580A is a mutation that removes a glycosylation site from the 18 amino acid insert

of COX-2 (Table 1a) (106). The Y371F mutant does not have cyclooxygenase activity because the tyrosine involved in the initial step in hydrogen abstraction has been changed to phenylalanine (Table 1a) (107). All the plasmids were used for stable transfection in Flp-In 293 cell lines.

Construction of Flag-tagged COX-2 in the pOSML vector. Flag-tagged COX-2 in pcDNA5/FRT/TO was directly subcloned into the *Not I* site of the pOSML vector (Appendix, Fig. 25) (Appendix, Table 8) and was used for the transient transfection of 293 Freestyle cells.

Cell culture. Frozen Sf21 cells were stored in liquid nitrogen until ready to use. Frozen vials were thawed in a 37°C water bath and transferred into a 125 mL shake flask containing 27 mL of pre-warmed Sf-900 II SFM, and incubated in a 27°C ± 0.5°C non-humidified, ambient air-regulated incubator in flasks. Sf21 cells were grown in shake flasks to $\geq 2 \times 10^6$ viable cells/ml, and infected at an M.O.I of 1:1, with baculovirus constructs. The cells were then grown for 72 h and harvested.

The Flp-In T-Rex -293 cell line constitutively expresses the *lacZ*-zeocin fusion gene, the Tet repressor and contains a single integrated Flp Recombination Target (FRT) site. The cell line can be used to generate tetracycline-inducible cell lines with high frequency by co-transfecting the pcDNA5/FRT/TO expression vector containing a gene of interest together with the Flp recombinase expression plasmid, pOG44 (Appendix, Fig. 26). Flp recombinase mediates insertion of the pcDNA5/FRT/TO expression construct into the genome at the FRT integration site through site-specific DNA recombination (Appendix, Fig. 27). Following the transfection, the Flp-In-TREx-293 expression clones should become sensitive to zeocin and should be selected with 100µg/ml hygromycin B to generate a stable cell line. Expression of the transfected gene can be induced with tetracycline.

Flp-In T-REX-293 cells (Invitrogen) were cultured in 100mm plates containing 10 ml of complete medium (DME high-glucose medium containing 10% fetal bovine serum, with 1% Pen-Strep containing 100μg/ml zeocin and 15μg/ml blasticidin) at 37°C with 6% CO_2. The cells were subcultured when 80-90% confluent (2-5 days). All medium was removed and the cells were washed once with 10 ml PBS. One ml 0.25% trypsin in versene (0.14 M NaCl, 1.5 mM KH2PO4, 2.7 mM KCl, 8.0 mM Na2HPO4, 0.5 mM EDTA) solution was then added and the cells were incubated for 1-5 minutes at room temperature until the cells detached. Nine ml of complete medium was then added and the solution was pipetted up and down to break up clumps of cells. One ml of the 10 ml cell suspension was transferred to a new 100mm plate and 10 ml fresh, complete medium containing zeocin and blasticidin was added.

The HEK 293 cell line is a permanent line established from primary embryonic human kidney transformed with sheared human adenovirus type 5 DNA. The E1A adenovirus gene is expressed in these cells and participates in transactivation of some viral promoters, allowing these cells to produce very high levels of protein. The FreeStyle™ 293 cell line is a variant of the 293 cell line that has been adapted to suspension growth in FreeStyle™ 293 Expression Medium. Cells were subcultured at approximately 1-1.5 $x10^6$ viable cells/ml, and were subcultured approximately every 48-72 hrs into new shaker flasks at 0.1-0.2 x 10^6 viable cells/ml. Flasks were incubated in a 37°C incubator containing a humidified atmosphere of 6% CO2 in air on an orbital shaker platform rotating at 135 rpm.

Isolation viruses from insect Sf21 cells with Flag-tagged COX-2-pFastBac and COX-2 *del*581-598-pFastBac. The BAC-to-BAC Baculovirus Expression System from Invitrogen was used to construct viruses to express the COX mutants in insect cells (Sf21, *Spodoptera frugiperda*). The pFastBac recombinants were first

transformed into MAX efficiency DH10Bac cells, and the recombinant bacmid DNA was isolated.

9 x 10^5 Sf 21 cells were plated in 2 ml of Sf-900 II SFM containing 50unit/ml penicillin and 50μg/ml streptomycin. Five μl of baculovirus DNA was diluted into 100 μl Sf-900 II SFM without antibiotics. Cellfectin was mixed and 6 μl was added separately into 100 μl of Sf-900 II SFM without antibiotics. The diluted DNA was combined with diluted Cellfectin, mixed gently and incubated for 15-45 minutes at room temperature. The growth medium was removed and the cells were rinsed with Sf-900 II SFM without antibiotics. The wash medium was removed and 0.8 ml of Sf-900 II SFM was added to the DNA-Cellfectin complex, mixed gently and added to the cells. Cells were incubated at 27°C for 5 hrs. The transfection mixture was removed and replaced with 2 ml of Sf-900 II SFM containing antibiotics. Cells were incubated at 27°C. After three days, the recombinant baculoviruses were harvested and used to infect Sf21 cells. These cells were harvested after three or four days by centrifugation at 5000 rpm for 10 minutes (Beckman TJ-6 centrifuge). The virus-containing supernatant was saved for amplification and the cell pellet was examined for protein expression.

Cyclooxygenase Assay Using the O_2 Electrode. Cyclooxygenase activity was measured polarmetrically as described previously (108). Cuvettes used to measure O_2 consumption were loaded with 3ml 0.1M Tris-HCl pH 8.0 containing 100μl 2mg/ml arachidonic acid, 1mM Phenol and 25μl 3.4mg/ml hemoglobin. The reaction was initiated by injecting homogenates of baculovirus-infected Sf21 cells. One unit of activity is the amount of enzyme required to convert 1nmole O_2 / min at 37^0C under the assay conditions described.

Stable transfection of different forms of cyclooxygenase in 293 Flp-In T-Rex system. pCDNA5/FRT/TO plasmids containing for the COX mutants were cotransfected with the Flp-recombinase expression vector pOG44 into Flp-In 293 cells (Invitrogen). One day before transfection, a 60mm plate was plated with 1 x 10^6 cells/4 ml in growth medium without antibiotics. Cells reached 90-95% confluence by the time of transfection. For each transfection, 8 μg DNA (7.2μg COX-pCDNA: 0.8μg pOG44) was diluted into 0.5 ml of Opti-MEM® Medium without serum and mixed gently. Lipofectamine™ 2000 (20 μl) was gently mixed in 0.5 ml Opti-MEM® I Medium and incubated for 5 minutes at room temperature. After 5 minutes incubation, the diluted DNA and diluted Lipofectamine™ 2000 were combined, mixed gently and incubated for 20 minutes at room temperature. The mixture was then added to a plate containing cells and medium and mixed gently by rocking the plate back and forth. Cells were incubated at 37°C in a CO_2 incubator for 24 hrs, and then the old medium was removed and fresh medium was added. After 48 hrs, cells were divided 1:5 into new plates with fresh growth medium. Selective medium containing 15μg/ml blasticidin and 100μg/ml hygromycin B was added after 2-3 hrs. Clones formed within two weeks and individual colonies were isolated with sterile Q-tips (Fisher). Cell lines were tested for inducible expression of COX proteins by treating the selected colony cells with 1μg/ml tetracycline for 24 hrs, and followed by Western blot analysis.

Transient tansfection of Flag-tagged COX-2 into FreeStyle 293 Suspension Cultures. Twenty four hrs before transfection, FreeStyle™293 cells were subcultured to 6~7 x 10^5 cells/ml in fresh FreeStyle™ 293 Expression Medium on an orbital shaker platform rotating at 135 rpm at 37°C at 6% CO2. On the day of transfection, the cell density is 1.2-1.5 x 10^6/ml. Twenty five ml of cells were added into each 125

ml shaker flask, and plasmid DNA (62.5 μg) was diluted into 150mM NaCl to a total volume of 1.25 ml. In a separate tube, 125 μl of polyethylenimine (PEI, 1mg/ml) (Polysciences, *Inc*) was diluted into 150mM NaCl to a total volume of 1.25 ml and mixed gently by inverting the tube. The plasmid DNA and PEI were immediately mixed and vortexed for 10 seconds. The DNA mixture was then incubated for 10 minutes at room temperature to allow complexes to form before adding to the cells. Transfected cells were incubated for 5 hrs at 37°C in 6% CO_2 atmosphere on an orbital shaker platform rotating at 135 rpm, and then 25ml of fresh FreeStyle™ 293 Expression Medium was added. Cells were harvested after 24 to 48 hrs and protein expression was examined by Western blot analysis.

Purification. COX-1 and COX-2 protein complexes were purified from stably-transfected 293 T-Rex cell lines that had been induced with tetracycline or from transiently transfected FreeStyle 293 cells. Cells were washed once with PBS, collected by centrifugation and homogenized in 0.5 ml lysis buffer (50mM Tris HCl, pH 7.4, 150 mM NaCl, 1mM EDTA) containing a cocktail of protease inhibitors (Complete-Mini Protease Inhibitors, Roche). The lysates were sonicated twice for 20 sec, and 1% Tween 20 was added. The homogenate was centrifuged at 3000× *g* for 15 min, and the supernatants were clarified by passing them through a 0.22 μm filter to remove particulate matter. Cleared cellular lysates were incubated with anti-Flag M2 agarose gel (Sigma) (10μl per 100mm plate) previously equilibrated in TBS (50mM Tris HCl, pH 7.4, 150 mM NaCl) with gentle mixing for 90 min. The resin was collected by centrifugation at 1000× *g* for 5 min and was washed with TBS buffer twice for 10 min. Flag-COX and associates proteins were eluted from the resin by incubation with 300 ng/μl 3xFlag peptide (Sigma) for 60 min at 4°C in 1ml TBS or 10 unit TEV protease (Invitrogen) in 150μl 1M Tris HCl (pH 8.0) with 10mM EDTA

overnight. In the initial experiments, beads were washed by Gly-HCl (pH 3.5) for 5 minutes to elute all the rest of the proteins that were attached in order to test the efficiency of elution.

Coomassie Staining of SDS-PAGE gels. Electrophoresis on 4-12% Bis-Tris NuPAGE polyacrylamide gels (Invitrogen) was used to fractionate the proteins purified by FLAG affinity chromatography and Coomassie Blue Stain was used to visualize the isolated proteins. Coomassie Brilliant Blue R250 (0.25% w/w) was dissolved in 90% methanol with 10% glacial acetic acid. The gel was immersed in five volumes of staining solution and placed on a slowly rotating platform overnight. The gel was destained by soaking in a 40% methanol/10% acetic acid solution on a slowly rocking platform until the background stain was gone and bands were clearly visible.

Western Blotting. Proteins samples for the Western blot were separated on a 4-12% Bis-Tris NuPAGE gels and transferred to nitrocellulose membranes. Visualization was performed by incubating with anti-flag (1:3000 dilution) and anti-COX (1:2000 dilution) primary antibody in 5% milk TBST (TBS plus 1% (v/v) Tween-20) solution for two hrs. The membranes were then washed four times with TBST for 10 minutes. Membranes were next incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies in 5% milk TBST solution (1:2000 dilution) for one hr. After four 10 minutes washes with TBST, immunoreactive proteins were visualized using the Western Lighting Chemiluminiscent Kit (Perkin Elmer, Boston, MA) and exposure to X-ray film (Amersham).

For the actin controls, the same blots were blocked a second time in 5% milk TBST overnight, and then incubated with anti-actin antibody (1:2000 dilution) in

TBST for two hs. The membrane was washed four times with TBST for 10 minutes, and the membranes were then incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody in 5% milk TBST solution (1:2000 dilution) for one hr. After four 10 minutes washes with TBST, actin was visualized as described above.

Mass spectrometry. Proteins isolated by a FLAG chromatography were electrophoresed into the stacking gel of an 8-16% Tris-HCl SDS-PAGE gel (Bio-Rad). The gels were fixed with a 40% methanol/20% acetic acid solution for 2 hrs, and then stained with Coomassie blue overnight. The next day, the gel was destained with a 10% acetic acid solution. The protein band was then excised by using a razor blade and sliced into 1-2mm pieces, all of which were placed into a single Eppendorf tube. The gel pieces were washed with 100 µl 100mM NH_4HCO_3 for 5 mins. The buffer was then replaced by 50µl of 100% acetonitrile and the gel pieces were dehydrated at room temperature for 15 minutes. Acetonitrile was then removed and the samples were dried completely in a Speedvac. The gel pieces were rehydrated with 50µl 10mM DTT in 100mM NH_4HCO_3 at 56°C for 30 minutes to reduce the proteins. DTT was then replaced with 100% acetonitrile and the gel pieces were incubated at room temperature for 5 minutes. This step was repeated, and the acetonitrile was removed and the samples were dried again in a Speedvac. 50µl of 55mM Iodoacetic acid (IAA) in 100mM NH_4HCO_3 was added. The samples were kept in the dark for 20 minutes at room temperature. The supernatant was discarded and the samples were washed briefly with 50µl 100mM NH_4HCO_3. The samples were then washed with new 100mM NH_4HCO_3 for 15 minutes at room temperature. The liquid was decanted and 50µl 100% acetonitrile was added at room temperature for 15 minutes. Next, the acetonitrile was removed and the samples were dried in a Speedvac. The samples were then rehydrated in 20µl digestion buffer (15ng/µl lyophilized trypsin in 50mM

NH_4HCO_3) and incubated for 45 minutes on ice. The excess digestion buffer was replaced with 20µl 50mM NH_4HCO_3 and incubated at 37°C overnight. The samples were then centrifuged at 15,000× g for 5 minutes. The liquid was collected in a new 1.5ml tube and set aside. 20µl of 60% ACN/ 1% TFA was added to each gel piece and sonicated for 10 minutes. The gel pieces were centrifuged and the supernatant was added to the previous supernatant. The gel pieces were washed twice and all of the liquid was combined and dried by using a Speedvac to less than 2µl. 18 µl MS buffer (1% Trifluoroacetic acid and 98% H_2O) was then added to the tubes and the tubes were sonicated for 5 minutes. The tryptic peptides were then injected onto a Paradigm Platinum Peptide Nanotrap (C18, 0.15 x 50mm). The bound peptides were eluted onto a 10 cm x 75 um New Objectives Picofrit column packed with Microm Magic C18 AQ packing material and eluted over 30 minutes with a flow rate of 250 nl/min and a gradient of 5% to 90% Acetonitrile, with constant 10% 1% formic acid in the first 24 minutes using a Michrom Paradigm liquid chromatography attached to a ThermoElectron LTQ Linear Ion trap mass spectrometer. The top five ions in each survey scan are then subjected to data-dependent low energy collision induced dissociation (CID). The resulting MS/MS spectra were converted to peak lists using BioWorks Browser v 3.2. All protein entries were downloaded from the National Center for Biotechnology Information web page (http://www.ncbi.nlm.nih.gov/, downloaded 01/26/2006) and the peak lists were searched against this library using Mascot. Identifications were considered positive if 2 peptides per protein were identified with a significant Mascot score ($p < 0.05$).

Results

Expression of Flag-tagged COX2 and COX-2 *del*581-598 in Sf21 cells. To determine if deletion of the 18 amino acid C-terminal cassette and the insertion of FLAG-TEV sequence at the N-terminus of cyclooxygenase-2 affected its activity, and to optimize methods for the affinity purification of Flag-tagged COX protein using FLAG affinity chromatography, Flag-tagged COX-2 and COX-2 *del*581-598 were expressed in Sf21 cells.

Polarmetric oxygen electrode assays of cyclooxygenase activity in cell crude lysates from individual infections demonstrated that each of these enzymes had activities similar to the untagged COX-2 N580A (a mutant form of COX-2 lacks the N580 C-terminal glycosylation site but has the similar activity as COX-2) (Table 1) (109). Neither the insertion of the FLAG-TEV sites or the deletion of the 18 amino acids affected the COX activity, validating the use of Flag-tagged COX-2 and COX-2 *del*581-598 in the mammalian cell studies.

Proteins	**Activity (μmole O_2/min/mg sample)**
COX-2 N580A	248
FLAG-TEV-COX2	215
FLAG-TEV-COX-2 *del*581-598	223
Sf21 cells only	0

Table 1. Comparison of the expression level of different COX-2 mutants in Sf21 insect cells. COX-2 N580A, a mutant form of COX-2 with similar activity as native COX-2 was used as a positive control.

Since our goal was to affinity-purify COX-2 complexes using the Flag-tagged COX-2, we first optimized the purification of Flag-tagged COX-2 from insect cells using anti-Flag agarose with 3X Flag peptide to elute COX-2. Western blotting with anti-Flag antibody and Coomassie staining was performed to check the efficiency of the purification (Fig 10a, b). Both Western blotting and Coomassie staining revealed bands migrated at approximately 72 kDa, which is the molecular weight of COX-2. No significant difference was observed in the migration of the Flag-tagged and un-tagged COX-2. Bands visible at molecular weights above 100 kDa are likely dimers and trimers of COX-2. The results showed that Flag-tagged COX-2 and COX-2

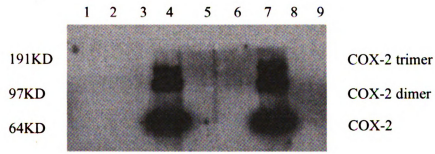


Figure 10(a). Western blot of Flag-tagged COX-2 and COX-2 *del*581-598 eluted with 3XFlag peptide. 15 μg of proteins were loaded onto each lane of a 10% NuPAGE Bis-Tris gel and proteins were separated by electrophoresis. After transferring to nitrocellulose membrane, Flag-tagged COX-2 and COX-2 *del*581-598 were detected by a specific anti-flag antibody. *Lane1-3* are purification fractionation samples isolated from COX-2 N580A infected insect cells: eluted with 3X Flag peptide in pH 7.4 TBS, flow through and eluted by pH 3.5 Gly-HCl respectively (see purification in methods). COX-2 N580A doesn't have a Flag-tag so no protein was observed in *Lane1-3*. *Lanes 4-6* are purification fractionation samples isolated from Flag-tagged hCOX2 infected insect cells: eluted with 3X Flag peptide in pH 7.4 TBS, flow through and eluted by pH 3.5 Gly-HCl respectively. *Lanes 7-9* are purification fractionation samples isolated from COX-2 *del*581-598 infected insect cells: eluted with 3X Flag peptide in pH 7.4 TBS, flow through and eluted by pH 3.5 Gly-HCl respectively.

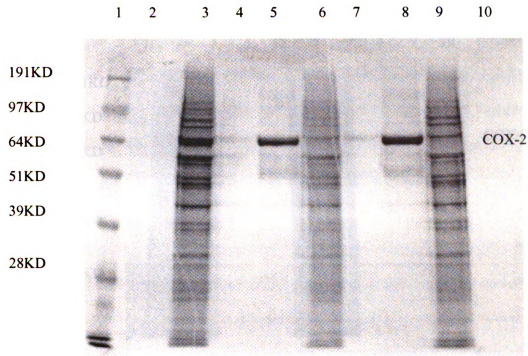


Figure 10(b). Coomassie Blue Stained PAGE gel of Flag-tagged COX-2 and COX-2 *del*581-598 eluted with 3X Flag peptide. 15 μg samples were loaded onto each lane of a 10% NuPAGE Bis-Tris gel and proteins were separated by electrophoresis. *Lane1* is Marker. *Lane2-4* are fractionation samples isolated from COX-2 N580A infected insect cells: eluted with 3X Flag peptide in pH 7.4 TBS, flow through and eluted by pH 3.5 Gly-HCl respectively (see purification in methods). COX-2 N580A doesn't have a Flag-tag so protein was only observed in *Lane 3*. *Lanes 5-7* are fractionation samples isolated from Flag-tagged COX-2 infected insect cells: eluted with 3X Flag peptide in pH 7.4 TBS, flow through and eluted by pH 3.5 Gly-HCl respectively. *Lanes 8-10* are fractionation samples isolated from COX-2 *del*581-598 infected insect cells: eluted with 3X Flag peptide in pH 7.4 TBS, flow through and eluted by pH 3.5 Gly-HCl respectively.

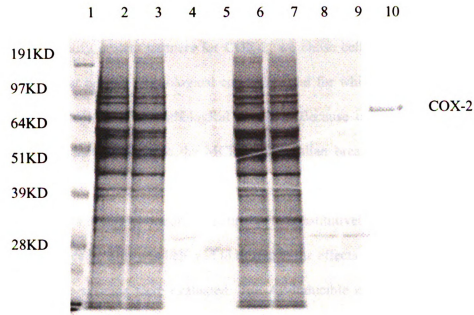


Figure 11. Coomassie Blue Stained PAGE gel of Flag-tagged COX-2 eluted with TEV protease. 25 μg of samples were loaded onto each lane of a 10% NuPAGE Bis-Tris gel and proteins were separated by electrophoresis. *Lane1* is Marker. *Lanes 2-5* are fractionation samples isolated from COX-2 N580A infected insect cells: crude lysate, flow through, eluted with TEV protease and eluted by pH 3.5 Gly-HCl respectively (see purification in methods). *Lanes 6-10* are fractionation samples isolated from Flag-tagged COX-2 infected insect cells: crude lysate, flow through, eluted with 25μg TEV protease, eluted with 30μg TEV protease and eluted by pH 3.5 Gly-HCl respectively.

*del*581-598 expressed to high levels in Sf21 cells and eluted efficiently from the anti-Flag agarose using 3X Flag peptide. Although the constructs contained TEV protease sites adjacent to the Flag-tag, no COX-2 elution was observed upon treatment with TEV protease. However, the COX-2 could be released at low pH using Glycine-HCl buffer (pH 3.5) (Fig 11). Longer incubations with TEV protease and increasing the

concentration of the protease didn't result in any significant release of the COX-2. The Flag sites binding to the anti-Flag beads maybe sterically block the TEV site.

Expression of Flag-tagged COX2 and COX-2 *del*581-598 in MCF-7 cells. When trying to identify protein partners for COX-2, we chose cell lines that might express COX-2 under normal physiological conditions and for which COX-2 might have an important biological or pathological function. Because of our interest in COX-2 for breast cancer, we chose the MCF-7 mammalian breast cancer-derived epithelial cell line.

Previous studies in this laboratory were unable to constitutively express COX-2 stably in transfected cells. This was believed to be due to the effects of COX-2 on cell growth, so inducible systems were evaluated. The AP-inducible expression system (ARIAD Pharmaceuticals) was the first system we attempted to use to construct an inducible COX-2 expressing cell line. This system uses a chemical inducer (AP) to link the activation and DNA binding domains of a bipartite transcription factor to form a functional transcription factor that stimulates transcription of a target gene from a synthetic promoter (Appendix, Fig 28a). AP (AP21967) is a chemically modified derivative of rapamycin that can be used to induce heterodimerization of engineered proteins. AP21967 has two separate motifs that can each bind with high affinity to the transcription activation and DNA binding protein modules (Appendix, Fig 28b). This system is one of the most tightly controlled regulated expression systems yet developed (110).

Flag-tagged COX-2 and COX-2 *del*581-598 were cloned into the target Ariad gene expression vector pLH-Z_{12}I-PL (Appendix, Fig 28c) and transfected into a MCF-7 cell line expressing the Ariad bipartite transcription factor (Appendix, Fig 28d).

Colony number	Activity/100mm plate
1	1.17
2	0.92
3	11.45
4	25.48
5	333.70
6	0.90
7	0.93
8	0.98
9	141.20
10	0.89
11	0.78
12	1107.00
13	12.15
14	0.06
15	0.96
16	0.71
17	0.91
18	1.29
19	388.60
20	1.06
21	1658.00
22	1.09
Cells only	0.34

Table 2. Luciferase Assay for individual colonies of stably transfected MCF-7 cells

This parent cell line was developed by the Gallo and Conrad laboratories at Michigan State University and has been used for expression of a Flag-tagged version of MLK-3 (111) and estrogen receptor (unpublished data). To verify that the system worked in our hands, luciferase was transfected as a positive control. Luciferase activity (Appendix, Fig 29) was tested according to the manufacturer's protocol (Sigma). Significant inducible luciferase activity was detected in 8 of 22 colonies (Table 2) which verified the integrity of the parent cell line and our experimental technique. COX-2 expression was also screened in stable colonies by Western blot analysis. Surprisingly, no expression could be detected for Flag-tagged COX-2 or COX-2 *del*581-598 in any of the antibiotic resistant colonies. Numerous transfections were tested as well as several different COX-2 antibodies, all with negative results.

There is no simple explanation as to why Flag-tagged COX-2 and COX-2 *del*581-598 could not be expressed in the AP system. The high sensitivity of the luciferase assay may simply allow detection of lower level of luciferase compared to the Western detection of COX-2 in this cell system. As this cell system is very tightly regulated, it does not seem likely that leaky expression of COX-2 played a role in our inability to isolate protein-expressing colonies.

Isolation of colonies that inducibly express cyclooxygenase in 293 Flp-In T-Rex cell system and identifying possible partners. Since we were unsuccessful using the AP system to express COX-2, we next tried the tetracycline-inducible 293 Flp-In T-Rex system. Colonies selected from different transfections were checked by Western blot for protein expression. Although the efficiency of recovering antibiotic-resistant colonies that expressed cyclooxygenase varied widely depending on the isozyme (COX-1 > COX-2) and the construct (Flag-tagged COX-2 < Flag-tagged COX-2 mutants), multiple cell lines that inducibly expressed Flag-tagged COX-2,

COX-2 *del*581-598, COX-1 and COX-1 *ins*580-598 were successfully isolated (Fig 12) (See Chapter III, Table 4). Respective colonies were grown (2 X 10^8 cells) and harvested 24 hr after tetracycline induction. A corresponding number of uninduced cells were isolated as negative controls. Protein homogenates from induced and uninduced cells were incubated with anti-Flag agarose beads to purify COX as described in the Methods section. The proteins isolated following the affinity purification were electrophoresed into the stacking gel of an 8-16% Tris-HCl SDS-PAGE gel (Bio-Rad), not to fractionate the proteins, but to remove any detergent that might interference with mass spectral analysis. The entire protein band was then excised and the proteins were digested from the gel with trypsin and then analyzed by mass spectrometry. Proteins were identified from mass spectral data by analysis using the Mascot program using the NCBI-NR database. Several criteria were used to filter those proteins identified by Mascot that might be relevant protein partners for COX-2.

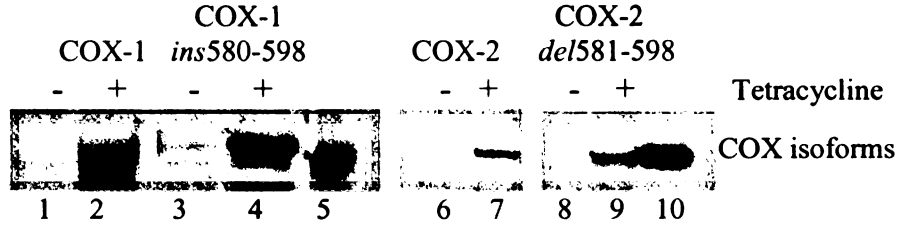


Figure 12. Western blot analysis of the inducible expression of COX isoforms in Flp-In 293 cells. Cells were treated with or without tetracycline. Lysates were separated on a 4-12% NuPAGE Bis-Tris gel, transferred to nitrocellulose membrane and visualized with a specific anti-flag antibody or an anti-COX antibody. *Lanes 1* and *2* was lysates isolated from Flag-tagged COX-1 expressing Flp-In 293 cells. *Lanes 3* and *4* was lysates isolated from Flag-tagged COX-1 *ins*580-598 expressing Flp-In 293 cells. *Lane5* shows a purified COX-1 protein standard. *Lanes 6* and *7* was lysates isolated from Flag-tagged COX-2 expressing Flp-In 293 cells. *Lanes 8* and *9* was lysates isolated from Flag-tagged COX-2 *del*581-598 expressing Flp-In 293 cells. *Lane10* shows a purified Flag-tagged COX-2.

Proteins identified in both control and induced cells were considered non-specific proteins that either bind non-specifically to the Flag-agarose beads, or were antibodies that detached from the anti-Flag agarose beads. For the proteins that were identified only in the isolated samples from cyclooxygenase-expressing cells, Mascot scores >37 were considered reliable using the NCBI-NR database. The higher the score, the more likely the protein identification is correct. Although multiple spectra are often assigned to a single protein identity, Mascot uses a system of red and bold typefaces to mark the difference. The first time Mascot assigns a peptide to a protein that peptide is shown in bold face. Whenever a spectrum of a peptide is the top ranking peptide match for a protein, it is shown in red. On the other hand, if the peptide is not shown in bold, this spectrum may also represent a peptide of another protein. If the peptide is black this indicates a lower fitness of match. The more bold and red peptide matches a protein is identified with, the more reliable it becomes. For our analyses, only protein having a mascot score above 37 using NCBI-NR database with single peptide identified more than once or multiple peptide identifications, at least one of which was typefaced in bold red, were considered as reliable protein identifications.

Protein partners for Flag-tagged COX-2 in 293 Flp-In T-Rex cells. Since the expression level of native Flag-tagged COX-2 in 293 Flp-In T-Rex cells was low, it was difficult to obtain sufficient protein for Mass spectrometry analysis. Of seven experiments conducted to identify COX-2 protein partners, five of them successfully identified COX-2. Only proteins that were identified in the tetracycline-induced cells but not in the un-induced cells and met our criteria were considered the potential partners.

BiP was identified as the second highest scoring protein (219) in the first experiment (COX-2 with the highest Mascot score (629) and 29 peptides) (Table 3 (a-

Proteins	Score	Peptides	Unique Peptides	NCBI-G.I.	Function
COX-2	629	29	9	181254	Catalyze the committed step in prostaglandins synthesis
BiP	219	4	3	6470150	Facilitate the assembly of multimeric protein complexes inside the ER
Heat shock-induced protein	130	2	2	188492	Molecular chaperones
Heat shock protein 90	95	2	2	67462296	Molecular chaperone
Heat shock 70 kDa protein 7	64	2	2	1346317	HSP70 family
FAM44A	53	2	1	Q8NFC6	Containing AT-hook DNA binding motif
Chaperonin	47	3	2	306890	HSP60 family: posttranslational modification, protein turnover, chaperones

Table 3(a-1). Proteins co-purified with Flag-tagged COX-2 in 293 Flp-in cells (experiment 1). Protein homogenates were purified with anti-Flag agarose beads. The proteins isolated were electrophoresed into the stacking gel of an 8-16% Tris-HCl SDS-PAGE gel. The entire protein band was excised and digested out of the gel with trypsin to be analyzed by mass spectrometry. Only proteins had a Mascot scores above 37 with single peptide identified more than once or multiple peptides identifications and at least one of which was bold red were listed.

1)). BiP is a 78 kDa glucose-regulated protein homolog precursor. This protein is a chaperone-like protein that facilitates the assembly of multimeric ER protein complexes (112). COX-2 and BiP protein both localize to the ER membrane

indicating that BiP could be involved in assisting the folding of COX-2. With three unique peptides and a high Mascot score, the identification seems likely to be correct. But this was the only experiments in which this protein was identified. It could be an artifact due to over-expression of COX-2 in this cell line, or an anomaly of the isolation in this experiment.

Four heat shock proteins were identified in the induced cells in the first experiment: Heat shock-induced protein, Heat shock protein 90, Heat shock 70 kDa protein 7 and Chaperonin. The scores varied from 47-130 and only one or two unique peptides were identified for each protein. Normally heat shock proteins act like chaperones for protein folding (113). However, only the Heat shock-induced protein (GI/188492) was identified in more than one experiment. Given that different but similar heat shock proteins were also identified in the proteins purified from the control cells, it may be that these proteins are purified non-specifically and their similar sequences lead to variable identification by the Mascot program.

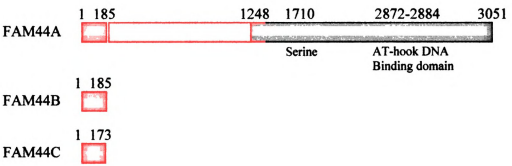


Figure 13. Sequence alignment of FAM44 proteins. Ser1710 is targeted for phosphorylation by ATM and ATR upon DNA damage. AT-hook DNA binding domain starts at site 2872 containing a GRP tripeptide.

FAM44A was another protein that was identified only in the induced cells with a score of 53 and 1 unique bold red peptide. Its cDNA was isolated from human chromosomes 4 (114). FAM44 protein has 3 members: FAM44A, FAM44B and FAM44C which are coded from chromosome 4, 5, 18 respectively. FAM44B has been reported to be involved in chromosome segregation and mitosis (115). Protein sequence alignment demonstrates that FAM44B and FAM44C are most closely related (75% identity). The N terminus of FAM44A is very similar to FAM44B and FAM44C. However FAM44A is much larger than other family members with a large C-terminal extension (Fig 13). FAM44A protein has AT-hook DNA binding domain which is prevalent in many eukaryotic nuclear proteins (116). A number of experiments have demonstrated that AT-hook-containing proteins like HMG-I(Y) play important roles in chromatin structure and act as transcription factor cofactors (117-119). AT-hook containing proteins ELYS/MEL-28 have also been reported as nuclear envelope proteins for nuclear pore assembly and proper cell division (120-122). So it is possible that FAM44A protein may localize to the nuclear membrane and function in the gene transcription regulation. (Subcellular location of COX-2 is also in the nuclear envelope.) FAM44A has also been reported that can be phosphorylated upon DNA damage, probably by ATM (ataxia telangiectasia mutated) or ATR (ATM and Rad3 related) (123,124). Proteins phosphorylated during DNA damage can serve as a cell cycle check-point control (125,126). For examples, tumor suppressor protein p53 was phosphorylated during DNA damage which prevented the degradation of the protein. The activated form of p53 can induce cell cycle arrest, activate DNA repair, or initiate apoptosis (125). Thus the interaction between COX-2 and FAM44A could be an alternative way to affect cell cycle. After binding, COX-2 may prevent FAM44A binding to its target DNA, disrupt its DNA repairing function

up DNA damage, promotes tumor growth. Another possibility is that COX-2 may interact with FAM44A for transcription regulation. As mentioned before, nuclear receptors, PPARs, may regulate gene expression by binding to prostanoids (91-93). However such a mechanism requires the nuclear transport of prostaglandins. FAM44A may serve as a chaperon for the COX-2-derived prostanoids to import into the nuclear to bind its target receptors for different gene expression regulation, or FAM44A may couple with COX-2-derived prostanoids to regulate gene transcription.

In experiments 4 and 5, only COX-2 protein met our criteria for a reliable protein identification (FAM44A was also identified but did not meet our cut-off criteria) (Table. 3(a-2)).

In experiment 6, COX-2 had a score of 186 and three unique bold red peptides, no other proteins were identified that met our criteria (FAM44A was also identified but did not meet our cut-off criteria)) (Table. 3(a-2)).

An experiment in which Kif was added to cell along with tetracycline, to stabilize COX-2 also yielded no new protein identification. In this experiment, COX-2 did have a Mascot score of 1402 with 56 peptides isolated and FAM44A was identified that met our criteria (Table. 3(a-3)).

Experiment Number	Proteins	Score	Peptides	Unique Peptides	NCBI-G.I.	Function
4	hCOX-2	92	2	2	181254	Catalyze the committed step in prostaglandins synthesis
5	hCOX-2	53	2	2	181254	Catalyze the committed step in prostaglandins synthesis
6	hCOX-2	186	6	3	181254	Catalyze the committed step in prostaglandins synthesis

Table 3(a-2). Proteins co-purified with Flag-tagged COX-2 in 293 Flp-in cells (experiment 4-6). Protein homogenates were purified with anti-Flag agarose beads. The proteins isolated were electrophoresed into the stacking gel of an 8-16% Tris-HCl SDS-PAGE gel. The entire protein band was excised and digested out of the gel with trypsin to be analyzed by mass spectrometry. Only proteins that had a Mascot scores above 37 with single peptide identified more than once or multiple peptides identifications and at least one of which was bold red were listed.

Proteins	Score	Peptides	Unique Peptides	NCBI-G.I.	Function
hCOX-2	1402	56	15	181254	Catalyze the committed step in prostaglandins synthesis
FAM44A	68	5	2	Q8NFC6	Containing AT-hook DNA binding motif

Table 3(a-3). Proteins co-purified with Flag-tagged COX-2 in 293 Flp-in cells (experiment 7). Protein homogenates were purified with anti-Flag agarose beads. The proteins isolated were electrophoresed into the stacking gel of an 8-16% Tris-HCl SDS-PAGE gel. The entire protein band was excised and digested out of the gel with trypsin to be analyzed by mass spectrometry. Only proteins that had a Mascot scores above 37 with single peptide identified more than once or multiple peptides identifications and at least one of which was bold red were listed.

Proteins	Score	Peptides	Unique Peptides	NCBI-G.I.	Function
hCOX-2	1010	34	14	181254	Catalyze the committed step in prostaglandins synthesis

Table 3(b). Proteins co-purified with Flag-tagged COX-2 *del*581-598 in 293 Flp-in cells. Protein homogenates were purified with anti-Flag agarose beads. The proteins isolated were electrophoresed into the stacking gel of an 8-16% Tris-HCl SDS-PAGE gel. The entire protein band was excised and digested out of the gel with trypsin to be analyzed by mass spectrometry. Only proteins that had a Mascot scores above 37 with single peptide identified more than once or multiple peptides identifications and at least one of which was bold red were listed.

Protein partners for COX-2 *del*581-598 in 293 Flp-In T-Rex cells. This experiment was also repeated for three times. COX-2 was the only unique protein identified (Table 3-b). The identification of proteins that specifically co-purified with COX-2 and not with COX-2 *del*581-598 suggests that the 18 amino acids may be important in protein-protein interaction.

Protein partners for COX-1 in 293 Flp-In T-Rex cells. Potential protein partners for native oCOX-1 were also identified by mass spectrometry analysis. Experiments were conducted twice and COX-1 was the only protein identified in the experiment one that met our criteria (Table. 3(c-1)). For experiments 2, several other proteins were identified besides the COX-1 protein. However all the other proteins identified had low scores (most peptides identified were black) and no common proteins were identified for COX-1 and COX-2 (Table. 3(c-2)).

Proteins	Score	Peptides	Unique Peptides	NCBI-G.I.	Function
COX-1	114	10	4	249624	Catalyze the committed step in prostaglandins synthesis

Table 3(c-1). Proteins co-purified with Flag-tagged COX-1 in 293 Flp-in cells (experiment 1). Protein homogenates were purified with anti-Flag agarose beads. The proteins isolated were electrophoresed into the stacking gel of an 8-16% Tris-HCl SDS-PAGE gel. The entire protein band was excised and digested out of the gel with trypsin to be analyzed by mass spectrometry. Only proteins that had a Mascot scores above 37 with single peptide identified more than once or multiple peptides identifications, at least one of which was bold red were listed.

Proteins	Score	Peptides	Unique Peptides	NCBI-G.I.	Function
COX-1	571	52	12	55958152	Catalyze the committed step in prostaglandins synthesis
MLL3	46	10	4	21427632	Belong to the ASC-2/NCOA6 complex (ASCOM), a coactivator complex of nuclear receptors
Protocadherin Flamingo 1	46	4	4	7407146	Similar to the secretin family of G-protein linked receptors
KIAA0543 protein	38	13	3	51466599	Regulation of transcription, DNA-dependent

Table 3(c-2). Proteins co-purified with Flag-tagged COX-1 in 293 Flp-in cells (experiment 2). Protein homogenates were purified with anti-Flag agarose beads. The proteins isolated were electrophoresed into the stacking gel of an 8-16% Tris-HCl SDS-PAGE gel. The entire protein band was excised and digested out of the gel with trypsin to be analyzed by mass spectrometry. Only proteins that had a Mascot scores above 37 with single peptide identified more than once or multiple peptides identifications, at least one of which was bold red were listed.

The Protocadherin Flamingo 1 had a Mascot score of 46. This protein is a seven-transmembrane receptor located on the cell surface (127), which is a different cell location compared to the location of COX-1, so it seems like this protein does not interact with COX-1 *in vivo*.

Myeloid/lymphoid or mixed-lineage leukemia protein 3 (MLL3, Histone-lysine N-methyltransferase, H3 lysine-4 specific MLL3) belongs to the ASC-2/NCOA6 complex (ASCOM) (128), a coactivator complex of nuclear receptors, involved in transcriptional coactivation. Since the subcellular location of COX-1 is not in the nuclear, this protein does not interact with COX-1.

KIAA0543 protein has a pretty low score of 38, but of 13 peptides, three unique ones were identified (129). The protein was present in the nuclear and may be involved in the transcriptional regulation. Again as with MLL3, it seems like the interaction does not occur in *vivo* and is an artifacts of the isolation.

Proteins	Score	Peptides	Unique Peptides	NCBI-G.I.	Function
COX-1	42	2	1	249624	Catalyze the committed step in prostaglandins synthesis

Table 3(d-1). Proteins co-purified with Flag-tagged COX-1 *ins*580-598 in 293 Flp-in cells (experiment 1). Protein homogenates were purified with anti-Flag agarose beads. The proteins isolated were electrophoresed into the stacking gel of an 8-16% Tris-HCl SDS-PAGE gel. The entire protein band was excised and digested out of the gel with trypsin to be analyzed by mass spectrometry. Only proteins that had a Mascot scores above 37 with single peptide identified more than once or multiple peptides identifications, at least one of which was bold red were listed.

Proteins	Score	Peptides	Unique Peptides	NCBI-G.I.	Function
COX-1	363	25	8	249624	Catalyze the committed step in prostaglandins synthesis
COX-2	101	6	2	181254	Catalyze the committed step in prostaglandins synthesis
FAM44A protein	52	8	1	Q8NFC6	Containing AT-hook DNA binding motif Homology of FAM44A
GRIA2 protein	47	5	3	14714846	Receptor for glutamate
Signal-induced proliferation-associated 1 like2	44	3	2	55664135	Containing a PDZ domain and a Rap-Gap domain
Zinc finger protein 469	44	21	3	51473106	May be involved in transcriptional regulation

Table 3(d-2). Proteins co-purified with Flag-tagged COX-1 *ins*580-598 in 293 Flp-in cells. Protein homogenates were purified with anti-Flag agarose beads. The proteins isolated were electrophoresed into the stacking gel of an 8-16% Tris-HCl SDS-PAGE gel. The entire protein band was excised and digested out of the gel with trypsin to be analyzed by mass spectrometry. Only proteins that had a Mascot scores above 37 with single peptide identified more than once or multiple peptides identifications, at least one of which was bold red were listed.

Proteins	Score	Peptides	Unique Peptides	NCBI-G.I.	Function
Beta actin variant	493	14	8	62897409	Essential for the structural integrity
Anti-colorectal carcinoma heavy chain	345	15	4	425518	Immunoglobulin C region
Chain M, Crystal Structure Of Fab Fragment Complexed With Gibberellin A4	326	23	5	24158784	Immunoglobulin domain variable region
Protein arginine N-methyltransferase 5	299	6	5	2323410	Methylates specific arginine residues in the small nuclear ribonucleoproteins Sm D1 and Sm D3
Ig kappa	138	19	3	227564	Immunoglobulin V region
Immunoglobulin kappa light chain	138	19	3	2970528	Immunoglobulin V region
Methylosome Protein 50	63	2	2	13559060	May regulate an early step in the assembly of U snRNPs, possibly the transfer of Sm proteins to the SMN-complex

Table 3(e). Proteins non-specifically isolated on anti-flag agrose beads for 293 Flp-in cells. Protein homogenates were purified with anti-Flag agarose beads. The proteins isolated were electrophoresed into the stacking gel of an 8-16% Tris-HCl SDS-PAGE gel. The entire protein band was excised and digested out of the gel with trypsin to be analyzed by mass spectrometry. This list contains the proteins that were isolated and identified in both samples, and represent what we assume are proteins that non-specifically bind anti-flag agrose beads. Only proteins that had a Mascot scores above 37 with single peptide identified more than once or multiple peptides identifications, at least one of which was bold red were listed.

Protein partners for COX-1 *ins*580-598 in 293 Flp-In T-Rex cell system. Potential protein partners for native oCOX-1 were also identified by mass spectrometry analysis. Experiments were conducted twice and COX-1 *ins*580-598 was the only protein identified in the experiment one that met our criteria (Table. 3(d-1)). For experiments 2, several unique proteins were identified only in the induced cell samples (Table 3-(d-2)). None of these proteins were co-isolated for both COX-1 and COX-1 *ins*580-598. Since COX-1 *ins*580-598 containing the 18-amino acids of COX-2 at its amino terminal, during the mass spectrometry analysis this peptide was recognized and identified as a match to COX-2 by Mascot. Interestingly one protein identified in COX-1 *ins*580-598 complex was FAM44A with a Mascot score of 53 and one unique peptide identified eight times.

FAM44A was identified with COX-2 repeatedly, and once with COX-1 *ins*580-598, but never with COX-2 *del*581-598 or COX-1, suggesting its interaction may dependent on the 18-amino acids insert.

Similar to oCOX-1, some proteins that were involved in DNA binding and transcription were also identified: such as Zinc finger protein 469. As stated before the interaction is probably not real.

Common artifactual protein identified in 293 Flp-In T-Rex cells. Common proteins present in all the COX expressing cells in the tetracycline-induced and uninduced cells are shown in Table 3-e.

Actin is one major protein that is identified. This protein is important for the cell structural. It could bind directly to the beads, causing an artifact (130).

A protein identified as anti-colorectal carcinoma heavy chain, Ig kappa, Immunoglobulin kappa light chain are probably eluted from the anti-Flag agarose beads.

Protein arginine N-methyltransferase 5 and methylosome protein 50 are both nuclear proteins. They likely bind to the agarose beads non-specifically.

Transient expression of Flag-tagged COX-2 in 293 Freestyle cell system and identifying possible partners. Transient transfections in the 293 Freestyle cells were also carried out to identify the possible partners for Flag-tagged COX-2 (Fig 14). In this system cells can be grown in the shaker flask instead of the plate, thus it is much easier to scale up more cells for the experiment. After transient transfection, cells were harvested, protein was purified with anti-Flag beads and analyzed by mass spectrometry (Table. 3-(f-1)).

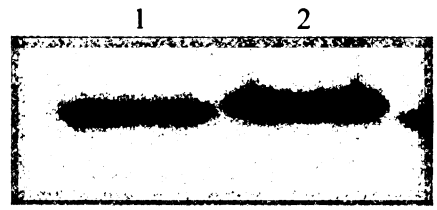


Figure 14. Western blot analysis of Flag-tagged COX-2 expression in 293 Freestyle cells. Lysates were separated on a 4-12% NuPAGE Bis-Tris gel, transferred to nitrocellulose membrane and visualized with a specific anti-flag antibody *Lanes 1* shows a purified Flag-tagged COX-2 standard. *Lane 2* was lysates isolated from transient transfection of Flag-tagged COX-2 in Freestyle 293cells.

Two heat shock proteins were identified only in the transfected cells: heat shock-induced protein and heat shock cognate 71 kDa protein. Heat shock-induced protein had the second highest score (521) and 20 peptides were identified. The heat shock-induced protein was the same protein identified in the T-Rex cells (Table. 3-a (1)). Since this protein was identified twice in different cell system both with high credibility, it could be a possible partner of COX-2. Recent work has proven that this

Proteins	Score	Peptides	Unique Peptides	NCBI-G.I.	Function
COX-2	546	36	10	181254	Catalyze the committed step in prostaglandins synthesis
Heat shock-induced protein	521	20	7	188492	In cooperation with other chaperones
KIAA0139	237	28	14	40788877	Motif in proteasome subunits, Int-6, Nip-1 and TRIP-15
DNA-binding protein TAXREB107	80	10	3	9802306	Ribosomal protein L6
Heat shock cognate 71 kDa protein	75	4	2	57085907	Chaperones
DEAD-box protein 3, Y-chromosomal	63	5	4	6014945	Inhibits the binding of survival motor neuron protein (SMN) to Sm proteins
Mammary tumor-associated protein INT6	62	2	2	2695641	Motif in proteasome subunits, Int-6, Nip-1 and TRIP-15
Splicing factor 3B subunit 3	54	4	2	19863446	Subunit of the splicing factor SF3B
FAM44A protein	53	5	3	Q8NFC6	Containing AT-hook DNA binding motif
EIF3S9 protein	52	4	4	12654669	Eukaryotic translation initiation factor 3 subunit 9

Table 3(f-1). Proteins co-purified with Flag-tagged COX-2 in 293 Freestyle cells (experiment 1). Protein homogenates were purified with anti-Flag agarose beads. The proteins isolated were electrophoresed into the stacking gel of an 8-16% Tris-HCl SDS-PAGE gel. The entire protein band was excised and digested out of the gel with trypsin to be analyzed by mass spectrometry. Only proteins that had a Mascot scores above 37 with single peptide identified more than once or multiple peptides identifications, at least one of which was bold red were listed

Proteins	Score	Peptides	Unique Peptides	NCBI-G.I.	Function
COX-2	1194	46	10	181254	Catalyze the committed step in prostaglandins synthesis
ATP synthase subunit beta, mitochondrial precursor	254	5	4	28940	Produces ATP from ADP in the presence of a proton gradient across the membrane. The beta chain is the catalytic subunit.
Calmodulin	128	3	1	71664	calcium-binding protein that can bind to and regulate a multitude of different protein targets
ATP synthase subunit alpha, mitochondrial precursor	48	2	1	4757810	Produces ATP from ADP in the presence of a proton gradient across the membrane. The alpha chain is a regulatory subunit.
Transmembrane protein 109 precursor (Mitsugumin-23)	44	2	1	13129092	May interacts with cytoplasmic protein(s) and participates in a housekeeping function

Table 3(f-2). Proteins co-purified with Flag-tagged COX-2 in 293 Freestyle cells (experiment 2). Protein homogenates were purified with anti-Flag agarose beads. The proteins isolated were electrophoresed into the stacking gel of an 8-16% Tris-HCl SDS-PAGE gel. The entire protein band was excised and digested out of the gel with trypsin to be analyzed by mass spectrometry. Only proteins that had a Mascot scores above 37 with single peptide identified more than once or multiple peptides identifications, at least one of which was bold red were listed.

protein can inhibit LPS-induced NF-κB signaling cascade activation and subsequently decrease COX-2 expression (131). Heat-shock induced protein can bind directly with TRAF-6 protein to inhibit its ubiquition. TRAF-6 will affect Iκk so it won't be activated, and thus NF-κB signaling cascade. So the interaction between COX-2 and the heat shock-induced protein could be an alternative way to affect the expression of COX-2. Heat shock cognate 71 kDa protein had a Mascot score of 76, although the four peptides were not identified with high confidence. This was the only time this protein was identified so the presence of the protein could simply be stimulated by the over-expression of COX-2 protein and thus be an artifact.

KIAA0139 isolated from 293 freestyle cells transfected with COX-2 have motifs involved in protein degradation. KIAA0139 had a score of 237 and 28 peptides, eleven of which were unique, indicated with high probability that this protein is present (although this was the only time this protein was identified) (132). COX-2 is degraded via the proteasome pathway. If the interaction was genuine, this protein could mediate the degradation of COX-2, especially if it is expressed at high level.

FAM44A protein was identified again with a score of 53, with five peptides, of which 3 were unique. This protein was identified in both the T-Rex and Freestyle cell system. FAM44A has been identified four times in the T-Rex cells. It was the only protein that was identified in both the native COX-2 complex and the COX-1 containing the COX-2 insert protein complex.

Several proteins that were involved in the transcription were identified: DNA-binding protein TAXREB107, DEAD-box protein 3, Y-chromosomal. Since the subcellular location of COX was not in the nuclear, it is a high probability that the interactions were not real.

The experiment was repeated once with twice the cells. Surprisingly no overlap proteins were found between the two transfections. Two proteins were newly identified as Mitochondrial F-type ATP synthase alpha and beta subunits respectively. The proteins are mitochondrial proteins located on the inner membrane and are involved in ATP synthesis (133). Both alpha and beta subunit belongs to the catalytic core F1. Thus, it is not clear why this protein might associate with COX-2.

Common artifactual protein identified in the 293 Freestyle cells. Immunoglobulin, anti-colorectal carcinoma heavy chain and immunoglobulin kappa chain, actin and the ribonucleoproteins are some common proteins that are both present in the transfected and control 293 cells (Table. 3g).

New artifactual proteins also showed up in this expression system: Ubiquitin and Histone 1. Why different artifactual proteins occur when COX-2 is purified using the 293 cells transient expression is not clear.

Proteins	Identification in Flp-In 293 cells/ Total experiments	Identification in Freestyle 293 cells/ Total experiments	Total identifications	NCBI-G.I.	Function
FAM44A	5/7	1/2	6	Q8NFC6	Containing AT-hook DNA binding motif
Heat shock-induced protein	1/7	1/2	2	188492	Molecular chaperones

Table 3(f-3). Proteins reproducibly co-purified with Flag-tagged COX-2.

Proteins	Score	Peptides	Unique Peptides	NCBI-G.I.	Function
Heterogeneous nuclear ribonucleoprotein C	1109	47	10	4758544	Binds pre-mRNA and nucleates the assembly of 40S hnRNP particles
Heterogeneous nuclear ribonucleoprotein K	1087	40	11	16923998	Binds pre-mRNA and nucleates the assembly of 40S hnRNP particles
Heterogeneous nuclear ribonucleoprotein A1	1027	43	9	133254	Binds pre-mRNA and nucleates the assembly of 40S hnRNP particles
Immunoglobulin kappa chain	732	29	3	1572705	Immunoglobulin V region
Histone 1, H2aj	568	30	5	7264004	Core component of nucleosome
Anti-colorectal carcinoma heavy chain	527	30	5	425518	Immunoglobulin C region
HNRPU protein	488	43	12	16041796	Binds to pre-mRNA
Vimentin	488	22	9	37852	Cytoskeletal protein, assembles into 10nm filaments
Heat shock protein	260	9	4	386785	HSP70 family
Dead box, X isoform	237	12	5	2580552	Helicase superfamily c-terminal domain
Beta actin variant	181	8	4	62897625	Essential for the structural integrity
Ubiquitin	139	7	3	340062	Mark other proteins for destruction

Table 3(g). Proteins non-specifically isolated on anti-flag agrose beads for 293 Freestyle cells. This list contains the proteins that were isolated and identified in both samples, and represent what we assume are proteins that non-specifically bind anti-flag agrose beads. Only proteins that had a Mascot scores above 37 with single peptide identified more than once or multiple peptides identifications, at least one of which was bold red were listed.

Discussion

FAM44A is the strongest candidate protein partner for COX-2. It was identified in 5 out of the 7 experiments in protein complexes with COX-2 purified from T-Rex 293 cells, and 1 in 2 of the experiments where COX-2 was expressed transiently in Freestyle 293 cells. FAM44A was also identified in protein complexes with COX-1 *ins*580-598 but never in the uninduced 293 cells, or in protein complexes with COX-1 or COX-2 *del*581-598, suggesting association requiring a specific interaction with the 18-amino acid cassette of COX-2. FAM44A protein have AT-hook DNA binding domain which is prevalent in many eukaryotic nuclear proteins (116) and a number of experiments have demonstrated that AT-hook-containing proteins play important roles in chromatin structure and act as transcription factor cofactors (117-119). AT-hook containing proteins ELYS/MEL-28 have also been reported as nuclear envelope proteins for nuclear pore assembly and proper cell division (120-122). So it is possible that FAM44A may localize to the nuclear membrane and function in the gene transcription regulation. FAM44A has also been reported that can be phosphorylated upon DNA damage, probably by ATM (ataxia telangiectasia mutated) or ATR (ATM and Rad3 related) (123,124). Proteins phosphorylated during DNA damage can serve as a cell cycle check-point control (125,126). Thus the interaction between COX-2 and FAM44A could be an alternative way to affect cell cycle. COX-2 has been previously reported involving in cell cycle control. NSAIDS can inhibit cell growth by inducing apoptosis and cell cycle arrest in either human hepatocellular carcinoma cells or human oesophageal adenocarcinima cells (134,135). Serum induced cell growth in NIH 3T3 cells was related to G_0 to G_1 cell cycle control by expressing COX-2 (136). Another possibility is that FAM44A may serve as a chaperon for import of COX-2-derived prostanoids to nucleus. For future study, co-

immunoprecipitation and GST pull down assay need to be carried out to confirm the interaction.

The heat shock-induced protein was another protein that was identified in both T-Rex and Freestyle systems associating with COX-2. It was identified 1 out of the 7 experiments in protein complexes with COX-2 purified from T-Rex 293 cells, and 1 in 2 experiments where COX-2 was expressed transiently in Freestyle 293 cells. Since heat shock-induced protein was not present in complexes with the COX-1 *ins*580-598 or the COX-2 *del*581-598, the interaction may require COX-2 protein domains in addition to the 18-amino acid cassette of COX-2. Heat shock-induced protein may aid in the processing of COX-2 or be involved in its degradation. Recent work has proven that this protein can inhibit LPS-induced NF-κB signaling cascade activation and subsequently decrease COX-2 expression (131), thus the interaction between COX-2 and heat shock-induced protein may also affect the expression of COX-2. Although heat shock-induced protein has been identified in both systems, overall it was only co-purified twice with COX-2 in 9 experiments. It could be an artifact due to over-expression of COX-2, or an anomaly of the isolation in a specific experiment.

BiP, KIAA0139 and Mitochondrial F-type ATP synthase were also co-purified once with COX-2 complex in all 9 experiments. BiP protein identified in T-Rex cells is a possible partner for COX-2 as may be involved in COX-2 folding in ER as a chaperone. KIAA0139 protein isolated from 293 freestyle cells transfected with COX-2 could be involved in the degradation of COX-2. ATP synthase is a mitochondrial enzyme and its interaction with COX-2 has no defined functions. Since all three proteins were not present in complexes with COX-1 *ins*580-598 or COX-2 *del*581-598, their interaction may require COX-2 protein domains in addition to the 18-amino acid cassette of COX-2. Although they were all identified with high credibility (high

Mascot score and many bold red peptides identified) one time with COX-2, considering it's the only time they were found, it was possible that they were just an artifact due to over-expression of COX-2.

Several other heat shock proteins were also identified once in the 293 T-Rex or Freestyle system. Given that different but similar heat shock proteins were also identified in the proteins purified from the control cells, it may be that these proteins are purified non-specifically and their similar sequences lead to variable identification by the Mascot program.

For COX-2 *del*581-598 construct, 3 experiments were conducted, but no unique proteins were ever identified in the T-Rex cells. The identification of proteins that specifically co-purified with COX-2 and not with COX-2 *del*581-598 suggests that the 18 amino acids may be important in protein-protein interaction.

Two experiments were carried out to identify potential protein partners for COX-1 in T-Rex cells. The first experiment failed to capture any proteins that met our criteria except COX-1. When the experiment was repeated, several unique proteins co-purified with COX-1. However the Mascot scores of these three proteins, MLL3, Protocadherin Flamingo 1 and KIAA0543, identified are near 37 (cut-off score) and most peptides identified were low credibility. Moreover they don't share subcellular location with COX-1. It is reasonable to assume that none of the proteins were real partners for COX-1 *in vivo*. No protein partners were identified in common for COX-1 and COX-2. Since COX-1 and COX-2 have very different biological functions, it is reasonable to assume that the specific interaction identified in COX-2 may cause different functions between COX isozyme.

Among the proteins co-purified with COX-1 *ins*580-598, FAM44A protein was the most probable protein partner candidate. As stated above, this protein was also co-

purified with COX-2 complex and may association with COX-2 specifically via the 18-amino acid cassette. Although several other proteins were also identified, none of them were co-isolated with both COX-1 and COX-1 *ins*580-598. Thus, these proteins are likely artifacts.

The explanation for why not many reliable candidates were identified could be due to the followings: the interactions are transient, or that the interaction of these proteins have low affinity and do not survive purification. Furthermore 293 cells do not normally express endogenous COX-2, and may not express protein partners for COX-2. The failure to obtain any new protein partners even when using the Kifnusenin suggested that either no proteins interact with COX-2 during the degradation or the interaction has low affinity so no potential partners survive during purification.

One limitation of this experimental approach is the non-specific purification of protein via agarose affinity chromatography. A large number of proteins were identified in both COX expressing and control samples). These non-specific proteins had high Mascot scores with multiple peptides identified. Because the signals of these proteins are so intense, they may mask the MS spectral signals from the true COX-2 protein partners.

To overcome these problems, different cell lines could be used instead of the 293 cells. The cells should endogenously express COX isozymes to guarantee that potential protein partners exist. Secondly, alternative tag could be employed for COX protein purification.

CHAPTER III

EFFECTS OF EXPRESSION OF CYCLOOXYGENASE PROTEINS ON CELL BIOLOGY

Summary

Our investigation also included exploring the role of the 18-amino acid cassette on cell biology. COX-2 variant without the 18-amino acid cassette or which were catalytically inactive were expressed in a higher percentage of transfectants and at higher protein levels than native COX-2, indicating that both catalytic activity and the 18-amino acid cassette were important mediators of the protein expression. Immunocytochemistry of COX isoforms was carried out to examine whether the 18 amino acids affects the subcellular localization of COX enzymes, and no differences were observed. All COX proteins localized to the nuclear envelope and ER membrane. Growth curves and agar assays were also conducted on T-REX 293 cell lines stably expressing Flag-tagged COX-2, COX-2 *del*581-598, COX-1 and COX-1 *ins*580-598, but no significant difference was observed cells expressing the different COX isoforms. The $T_{1/2}$ of Flag-tagged COX-2, COX-2 *del*581-598, COX-1 and COX-1 *ins*580-598 were determined and found to be similar to each other, indicating that the activity and the 18-amino acid cassette may not be important in the degradation process. However results in Smith's laboratory later showed that the FLAG tag at the N-terminus affected the turnover rate of COX enzymes. Proteins without FLAG tags were constructed and expressed in the 293 T-REX cell line. Surprisingly the non-tagged COX-2 *del*581-598 protein has a much longer $T_{1/2}$ (compared to COX-2) while the COX-1 *ins*580-598 protein has a shorter $T_{1/2}$ (compared to COX-1), indicating that 18-amino acid cassette is involved in the protein turnover regulation. Furthermore

COX-2 N580A also has a much longer $T_{1/2}$ than native COX-2, suggesting that the C-terminal glycosylation site (just before the 18-amino acid cassette) was also important in the turnover regulation of COX proteins.

Introduction

Prostaglandin Endoperoxide H synthase-1 and 2 (PGHS-1 and PGHS-2; also known as cyclooxygenase-1 and 2, COX-1 and COX-2) catalyze the committed step in prostaglandins synthesis. Cyclooxygenase-1 and 2 have many features in common while many studies show that the biological function is quite different between the two. COX-1 is constitutively expressed in most tissues and knock-out mouse studies have confirmed its responsibility in platelet aggregation (76) and parturition (77, 78). COX-2, however, is not expressed in most unstimulated tissues but is rapidly induced by growth factors, cytokines, and tumor promoters. COX-2 is responsible for ovulation, implantation (79), and neonatal development (80, 81). To date, many evidences have indicated a primary role for COX-2, rather than COX-1 in carcinogenesis (84, 85).

New data from the Smith laboratory has indicted that one role of the 18-amino acid cassette is to mediate entry of COX-2 into the ER-associated degradation (ERAD) system that transports ER proteins to the cytoplasm (74). Their studies found that the insertion of the 18-amino acid cassette into COX-1 can destabilize the protein while deletion of the 18-amino acid cassette in COX-2 stabilized the protein. Thus, the 18-amino acid cassette has a critical role in regulation of COX-2 stability. The Smith group has also proposed that the glycosylation of N^{580} and subsequent trimming of the core sugar $Glc_3Man_9GlcNAc_2$ complex is necessary for the entry of COX-2 into the

ER-associated degradation (ERAD) system based in part on the effects of Kifnusenin, an inhibitor of terminal fucosidases that stabilized the protein (74). The different regulation of COX protein stability could be important for their different biological activity.

In chapter II I provided evidence that it is difficult to isolate cell lines that express COX-2, but COX-1 can be expressed without problem. If the 18-amino acid cassette is involved in regulating the protein expression, the deletion of the cassette should allow COX-2 expression and insertion of the cassette should prevent such expression.

Recent work demonstrated that cotransfection of COX-2 and mPGES-1 (microsomal PGE synthase) into HEK293 cells can stimulate the production of PGE_2, and thus cell proliferation (137). Co-expression of COX-2 and mPGES-1 in HEK293 cells can also resulted in cellular transformation and tumor formation. This was accompanied by changes in the expression of a variety of genes related to proliferation, morphology, adhesion and the cell cycle such as RhoA and TRAF-1. We attempts to replicate their experiments and also to determine process by examining the cell growth in transfected cell lines expressing different forms of COX-1 and -2.

There has been no good explanation for the apparent redundancy of COX-1 and 2. These two enzymes are so similar that it is difficult to explain why their biological activities are different. 18-amino acid cassette of COX-2 has been proved to be an important factor in regulation the protein stability. The study of this Chapter is to verify the results and determine if it is also responsible for other distinct biological activities of COX-2.

Experimental Procedures

Materials. Flp-In-T-Rex-293 cell line was from Invitrogen. Dulbecco's modified Eagle medium (DMEM), Lipofectamine 2000, tetracycline, blasticidin, zeocin, hygromycin, and penicillin-streptomycin were from Invitrogen/Gibco. Fetal bovine serum (FBS) was from Atlas Biologicals. Cycloheximide and puromycin were obtained from Sigma-Aldrich.

Construction of pCDNA expression plasmids for COX-2, COX-2 *del*581-598, COX-2 N580A, oCOX-1 and COX-1 *ins*580-598. Untagged version of the COX-2-pCDNA, COX-2 *del*581-598-pCDNA and COX-2 N580A-pCDNA were constructed using FLAG-TEV-plasmids as templates by PCR mediated mutation. The Flag-tagged COX-1-pCDNA and COX-1 *ins*580-598-pCDNA were used as templates to make untagged version. All the plasmids were used for the stable transfection in FLP-In 293 cell lines. The primers, vector and the PCR conditions used to design the above mutants are shown in the 'Appendix' section (Table. 7).

Construction of the human microsomal PGES (PGE_2 synthase) in the pBABE vector. cDNA of human prostaglandins E synthase was amplified from pOTB7 vector (Appendix Fig. 30) by PCR. The fragment was cloned into Zero blunt TOPO vector (Invitrogen) (Appendix Fig. 31) and then into pBABE-puro vector (Appendix Fig. 32) at the *EcoR I* site. This plasmid was used to transfect Flp-In 293 cells that inducibly co-express one of the COX mutants.

Stable transfection of mPGES in Flp-In 293 cells with COX-1 and COX-2 expressing. The pBABE-puro plasmid coding for the PGE synthase enzyme was transfected into COX expressing Flp-In 293 cells (Invitrogen). One day before transfection, 1 x 10^6 cells were plated into a 60mm tissue culture plate in 4 ml of growth medium. Cells were 90-95% confluent at the time of transfection. For each

transfection, 8 μg DNA (8μg mPGES-pBABE) was diluted in 0.5 ml of Opti-MEM® I Medium without serum and mixed gently. Transfections were carried out as described previous. Clones expressing target proteins were selected by treating the cells with 15μg/ml blasticidin, 100μg/ml hygromycin B and 1μg/ml puromycin. Constitutive expression of the mPGES was analyzed by Western blot analysis.

Western Blotting. Proteins samples for the Western blot were separated on a 4-12% Bis-Tris NuPAGE gels and transferred to nitrocellulose membranes. Visualization was performed by incubating with anti-flag (1:3000 dilution), anti-COX (1:2000 dilution) or anti-mPGES (1:500 dilution) primary antibody in 5% milk TBST (TBS plus 1% (v/v) Tween-20) solution for two hs. The membranes were then washed four times with TBST for 10 minutes. Membranes were next incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies in 5% milk TBST solution (1:2000 dilution) for one h. After four 10 minutes washes with TBST, immunoreactive proteins were visualized using the Western Lighting Chemiluminiscent Kit (Perkin Elmer, Boston, MA) and exposure to x-ray film (Amersham).

For the actin controls, the same blots were blocked a second time in 5% milk TBST overnight, and then incubated with Anti-actin antibody (1:2000 dilution) in TBST for two hs. The membrane was washed four times with TBST for 10 minutes, and the membranes were then incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody in 5% milk TBST solution (1:2000 dilution) for one h. After four washes with TBST for 10 minutes, actin was visualized as described above.

Cell Growth Curve and Semi-soft Agar Assay. Cells were seeded at 1.0×10^4 or 5.0×10^4 cells/ ml (6.0×10^4 or 3.0×10^5 cells/ 20cm^2 plate) and cultured for 10 days.

Individual plates were harvested every 2 days and cell number was determined by counting with a hemocytometer.

Semi-soft Agar Assay: Cells (10^4 cells/ml) were suspended in cell culture medium containing 1% (w/v) low-melt agarose at 37°C and plated on 60-mm culture dishes. After culturing for 10 days at 37 °C in a 6% CO_2 incubator, colonies were counted. Colony size was determined by measuring five random colonies in each plate using a calibrated lens ruler.

Immunocytochemistry of COX-1 and COX-2 mutants. Stably-transfected HEK 293 T-REX cells were plated on glass coverslips in 6 well plates in medium with 2mg/ml tetracycline overnight. For staining, the cells were washed with phosphate buffered saline (PBS), fixed by incubating with 2% paraformaldehyde in PBS for 10 minutes, and then washed with PBS containing 10% calf serum in (138). The cells were next incubated with a Flag antibodies diluted 1:20 in PBS containing 0.2% saponin and 10% calf serum for 30 min. After washing in PBS containing 10% calf serum, the samples were incubated for 30 min with a 1:40 dilution of FITC-conjugated goat anti-mouse IgG in PBS containing 0.2% saponin and 10% calf serum. The samples were washed with PBS containing 10% calf serum and then rinsed with PBS. Finally, coverslips containing the stained cells were mounted with PermaFluor on slides and examined by fluorescence microscopy.

Determination of COX protein $T_{1/2}$. Stably-transfected HEK 293 TREX cells were cultured in 100mm× 20mm plates. Eight plates of each cell line, 10^7 cells per plate were seeded. One plate was used as a no tetracycline negative control, the rest were induced for cyclooxygenase expression by adding 2μg/ml tetracycline for 24 hrs. The cells were washed then with PBS and incubated with the medium containing 50μm cycloheximide for 0, 2, 4, 6, 8, 12, 24 hrs before harvesting. Cells were

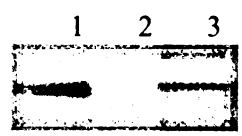
dissolved in 0.5ml PBS containing protease inhibitors (Complete Mini Protease Inhibitor, Roche) and sonicated for 3 times 10 seconds and expression of COX was determined by Western blotting. Expression levels were quatitated by analyzing the band density of the scanned X-ray film, using the Scion Image program (http://www.scioncorp.com/pages/scion_image_windows.htm). $T_{1/2}$ was calculated by integrated first-order velocity equation; $2.3\log [S]_0/[S] = kt$. $[S]_0$ represents the band density of the time 0; [S] represents the band density of the time 2 to 24 hrs respectively. All the band density was normalized against the actin control. The value of log [S] versus the time t (0-24 hr) was used to plot a straight line via Microsoft excel program, and the slope of the curve is $-k/2.3$ for equation $\log [S] = \log [S]_0 - kt/2.3$. When [S] equals to 1/2 $[S]_0$, then t in the equation is the $T_{1/2}$ of the protein $T_{1/2}$, and $T_{1/2} = -2.3\log 2/k$. Since k equals to -2.3slope from the curve, $T_{1/2}$ can be calculated by log2/ slope.

Ratio of expression and loss of expression. Cell clones expressing native COX-2 were much rarer than clones expressing other COX-2 mutants. To examine the reasons for lower native COX-2 expressing clones, different constructs of COX-1 and COX-2 were transfected, and the ratio of antibiotic resistant colonies expressing and not expressing each protein was determined. Twenty four or more antibiotic resistant colonies resulting from transfections of Flp-In 293 cells with expression plasmid constructs for COX-1 and COX-2 (Flag-tagged COX-2, COX-2 *del*581-598, COX-2 Y371F, COX-1 *ins*580-598, COX-1) were randomly selected. The effect of NS-398, a COX-2 selective inhibitor was also used to determine whether cyclooxygenase inhibitors would increase recovery of COX-2 expressing cell lines. Cells from each transfection were harvested and checked for expression by Western blotting.

The long term ability of stably transfected cells to express native COX-2 was also examined. Cells were cultured in the absence and presence of tetracycline to determine whether constitutive expression of COX-2 lead to loss of enzyme expression. After each harvest, cells were divided into collected. After 10 generations, protein expressions were checked by Western blot.

Results

Ratio of expression and loss of expression. Based on our difficulty expression COX-2 in MCF-7 cells and HEK 293 derived cell lines, we attempted to determine what structure or catalytic features of COX-2 prevented its expression. Transfection experiments with different forms of COX protein were carried out and the relative efficiency of isolating clones expressing different mutants was determined by Western blot (Table. 4). COX-1 and COX-1 *ins*580-598 were expressed in 100% of 24 randomly picked colonies. COX-2 had the lowest expression ratio, and was detectable in 26.7% of colonies. Expression ratios of COX-2 *del*581-598 and COX-2 Y371F



COX isoforms

Figure 15. Western blot examining stability of expression of COX in transfected cell lines. Samples were separated on a 4-12% NuPAGE Bis-Tris gel, transferred to nitrocellulose membrane and visualized with a specific anti-flag antibody. Lane *1* is standard Flag-tagged COX-2. *Lane 2* was lysates isolated from constitutive Flag-tagged COX-2 expressing Flp-in 293 cells. *Lanes 3* was lysates isolated form Flag-tagged COX-2 expressing Flp-in 293 cells respectively.

(an inactive mutant) were 41.7 and 45.8% respectively, higher than native COX-2 but lower than native COX-1 and COX-1 *ins*580-598.

Since inactive COX-2 (COX-2 Y371F) was expressed more readily than native COX-2, it is possible that COX-2 activity has a negative effect on cell growth. To test this possibility, the effect of addition of a COX-2 specific inhibitor NS398 (139) during the transfection process was tested to see if it increased our ability to isolate

Protein	Expressed colonies in all colonies	% Expressing
Flag-tagged COX-1	24/24	100.0
Flag-tagged COX-1 *ins*580-598	24/24	100.0
Flag-tagged COX-2	8/30	26.7
Flag-tagged COX-2+NS 398	11/12	91.7
Flag-tagged COX-2 *del*581-598	10/24	41.7
Flag-tagged COX-2 *del*581-598+NS 398	21/24	87.5
Flag-tagged COX-2 Y371F	11/24	45.8
Flag-tagged COX-2 Y371F +NS 398	21/24	87.5

Table 4. Transfection efficiency for COX proteins. Stably transfected Flp-In 293 cells line expressing different COX mutants were used to check the ratio of expression. Samples from different colonies were separated on a 4-12% NuPAGE Bis-Tris gel, transferred to nitrocellulose membrane and visualized with a specific anti-flag antibody. The ratio number of colonies that expressed the target protein was calculated.

COX-2 expression colonies. When NS398 was added during the selection process, 92% of colonies expressed native COX-2. Similar results were also obtained for the COX-2 *del*581-598 when treated with the drug.

Our results have shown that either deletion of the 18-amino acid cassette or inactivation of the enzyme could increase protein expression ratio as compared to the native COX-2, indicating both activity and the 18-amino acid cassette were important for the protein expression.

Experiments were also carried out to determine whether constitutive expression of COX-2 was detrimental to cell growth. If so, we expected to see a gradual loss of COX-2 expression overtime. As shown in Fig 15, expression of COX-2 disappeared in cells grown for 14 sub-culturing in the presence of tetracycline while non-constitutively COX-2 expressing cells maintained the normal expression. This further confirmed that COX-2 expression was tightly controlled in the cells.

Cell Growth Curve and Semi-soft Agar Assay for Flag-tagged COX-2, COX-2 *del*581-598, COX-1 and COX-1 *ins*580-598. Evidence has indicated that NSAIDs can reduce the incidence of colorectal cancers by inhibiting colon cancer cell growth and COX-2 was related to this effect (140). To determine if the COX enzymes can stimulate cell growth and if the 18-amino acid insert has a role in it, growth curve were carried out on T-Rex 293 cell lines stably expressing Flag-tagged COX-2, COX-2 *del*581-598, COX-1 and COX-1 *ins*580-598 (Fig 16(a-d)). Three separate experiments were conducted and similar results were obtained. No significant difference was observed either between the growths of uninduced and induced cells expressing the COX isoforms or between cells expressing the different COX isoforms.

As anchorage-independent growth is considered to be an *in vitro* test for tumorigenesis, we examined the growth of COX-transfected HEK293 cells in a

semisoft agar medium (Table. 4). No difference was found in the size and number of colonies between control cells and cells induced with tetracycline. Also, no difference was observed between different COX isoforms. As stated in Kudo *et al* (2003), cells expressing COX-2 alone formed small colonies when HEK293 parental cells did not grow appreciably in soft agar. In our case, 293 Flp-in cells can also form similar size and number of colonies as COX-2 expressing cells.

Since no differences were found in this case, we wonder if the difference was not significant enough to be observed. In order to magnify the growth difference mPGES was co-expressed with all COX isoforms in the next attempt.

Cell Growth Curve and Semi-soft Agar Assay for Flag-tagged COX-2, COX-2 *del*581-598, COX-1 and COX-1 *ins*580-598 with mPGES-1. The membrane-bound form of the PGE synthase (mPGES-1) has been previously reported to couple with COX-2, not COX-1, to produce PGE_2. PGE_2 can increase growth and motility of colorectal carcinoma cells through the EP4 receptor signaling pathway (141, 142). Expressing of PGES has also been reported to increase cell growth when co-expressed with COX-2 (134). To confirm these observations and also to evaluate the role of 18-amino acid insert in this process, we transfected mPGES into 293 T-Rex cell lines expressing different COX isoforms (Fig 17).

The Flag-tagged COX-2 and COX-2 *del*581-598 expressing cells grew faster than the control cells when co-expressed with mPGES (Fig 18(a-b)), probably by increasing PGE_2 production. On the other hand, even in cell lines constitutively expressing mPGES, no difference was observed in cell growth for COX-1 and COX-1 *ins*580-598 expressing cells compared to the control cells (Fig 18(c-d)). This could confirm the coupling between COX-2 and mPGES not COX-1. These results were in accordance to the results shown in Kudo *et al* (2003).

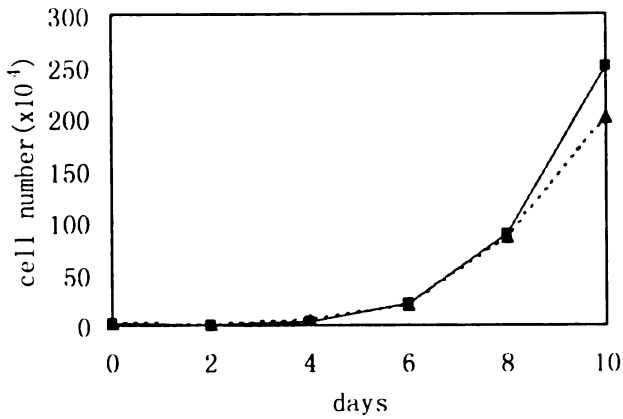
Although the cells expressing mPGES and COX-2 isoforms grew faster, both Flag-tagged COX-2 and COX-2 *del*581-598 had the same phenotype indicating that the 18 amino acids itself may not be involved.

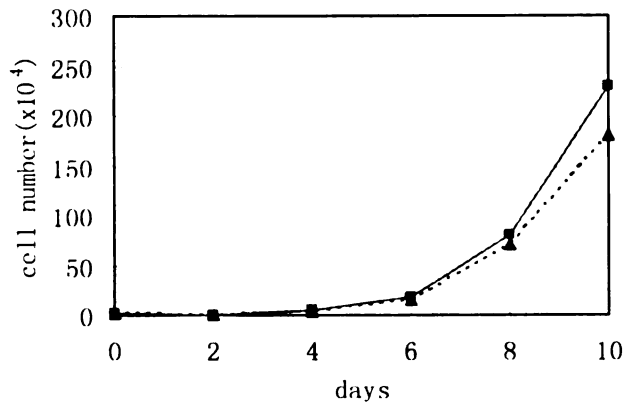
A colony-forming test was also done to check the phenotype of cells expressing different COX isoforms (Table 5). According to Kudo *et al* (2003), COX-2 and mPGES-1 coexpressing cells exhibited marked anchorage-independent growth, as manifested by the appearance of a number of large colonies. On the other hand, cells expressing COX-2 alone or cells co-expressing COX-1 and mPGES-1 formed fewer and smaller colonies (the size and number is about 5 times difference). However the results in our lab showed that no significant difference was found in the colony size and number between uninduced and induced cells expressing the COX isoforms or between cells expressing the different COX isoforms. This assay also confirmed that 18 amino acids were not involved in the cell growth.

Immunocytochemical localization of Flag-tagged COX-2, COX-2 *del*581-598, COX-2 Y371F, COX-1 and COX-1 *ins*580-598. To check if the 18-amino acid affects the subcellular localization of COX enzymes, immunocytochemistry of COX isoforms in transfected cells were conducted (Fig 19(a-e)). No differences were observed for any of the COX proteins: similar staining of the perinuclear envelope and ER membrane were observed for all COX isoforms.

The 18-amino acid cassette does not seem to regulate the subcellular localization of COX enzymes.

Determination turnover rates of Flag-tagged COX-2, COX-2 *del*581-598, COX-2 N580A, COX-1 and COX-1 *ins*580-598. Stably-transfected HEK 293 TREX cells were cultured and induced for cyclooxygenase expression by adding 2μg/ml tetracycline for 24 hrs. The cells were then incubated with the medium containing





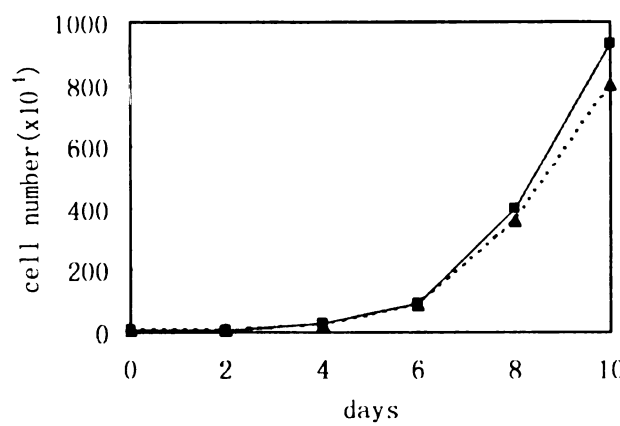


Figure 16((a)-1-3). Growth curve for 293 T-Rex cell lines expressing Flag-tagged COX-2 (Solid line is tetracycline induced cells and dash line is cell control. 1×10^{4} or 5×10^{4} cells were inoculated at day 0). Results are shown in three independent experiments.

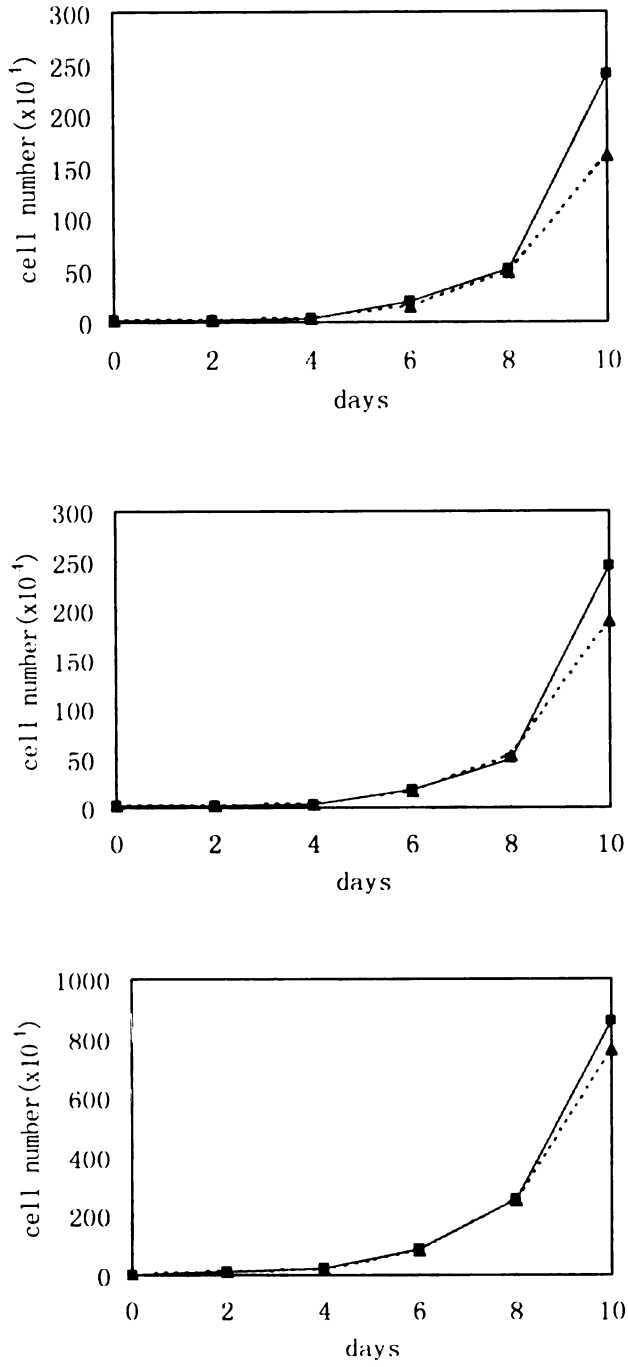


Figure 16((b)-1-3). Growth curve for 293 T-Rex cell lines expressing Flag-tagged COX-2 *del*581-598 (Solid line is tetracycline induced cells and dash line is cell control. 1×10^{4} or 5×10^{4} cells were inoculated at day 0). Results are shown in three independent experiments.

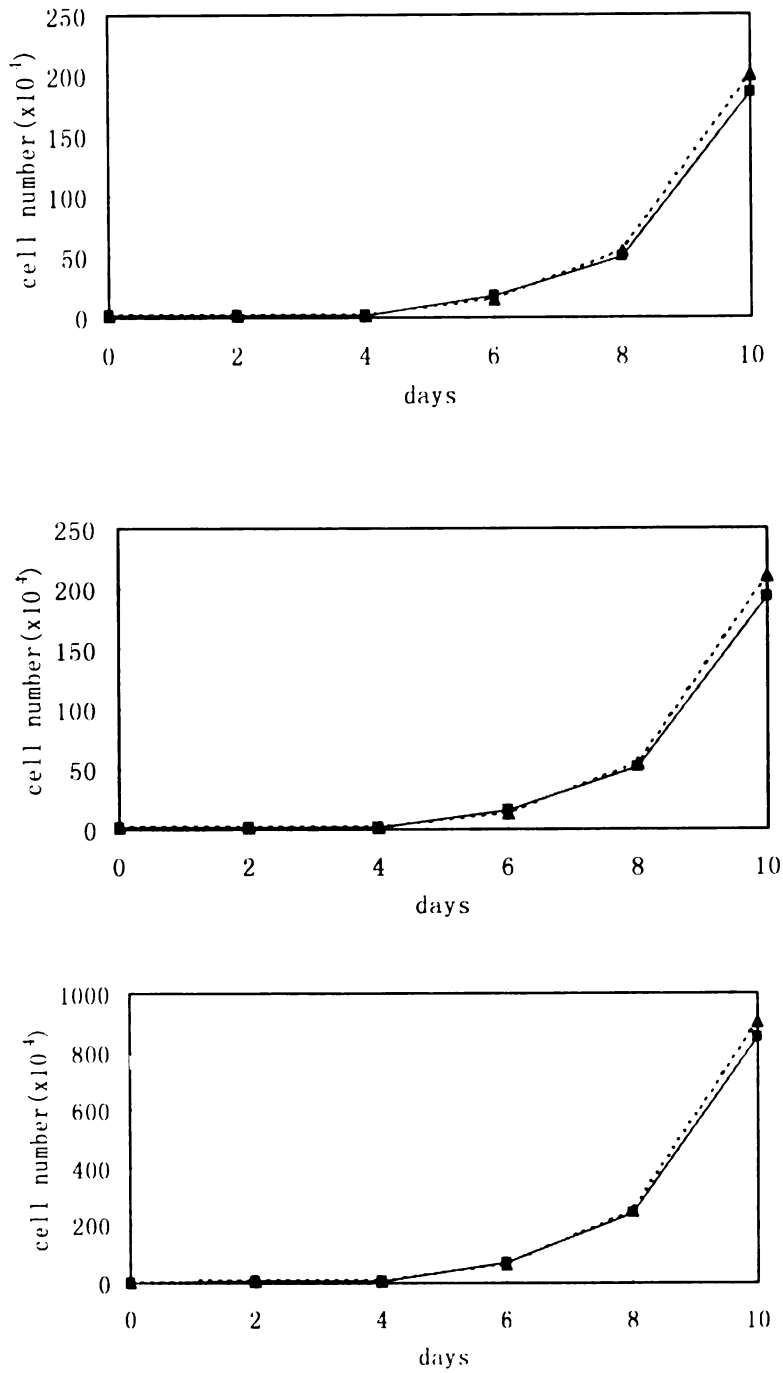


Figure 16((c)-1-3). Growth curve for 293 T-Rex cell lines expressing Flag-tagged COX-1 *ins*580-598 (Solid line is tetracycline induced cells and dash line is cell control. 1×10^{4} or 5×10^{4} cells were inoculated at day 0). Results are shown in three independent experiments.

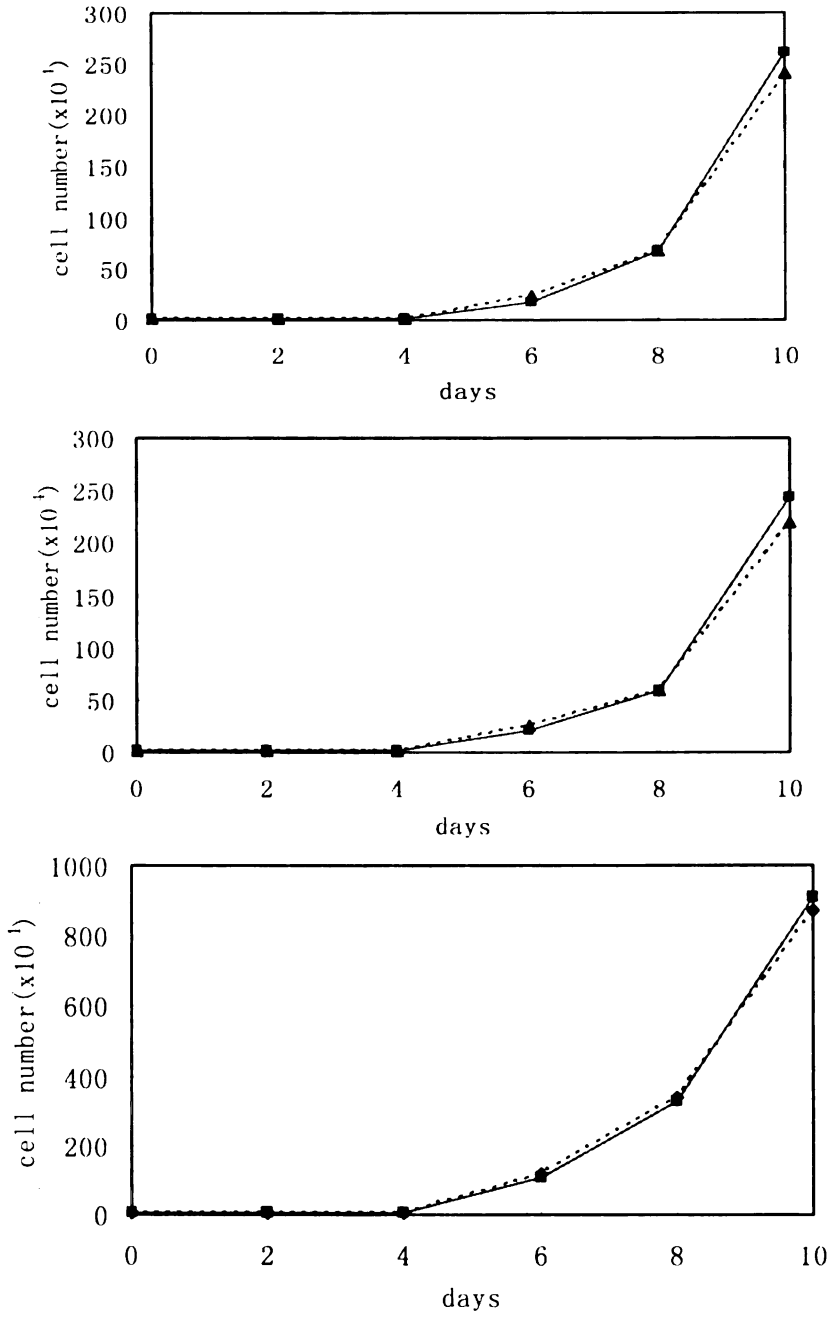


Figure 16((d)-1-3). Growth curve for 293 T-Rex cell lines expressing Flag-tagged COX-1 (Solid line is tetracycline induced cells and dash line is cell control. 1×10^{4} or 5×10^{4} cells were inoculated at day 0). Results are shown in three independent experiments.

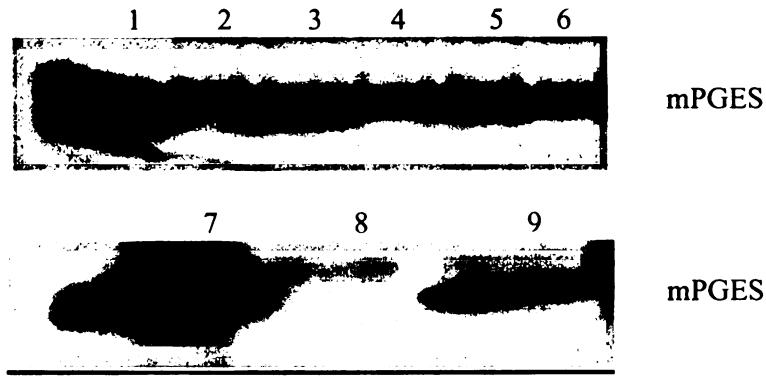


Figure 17. Western blot examining stability of expression of mPGES in COX expressing Flp-In 293 cell lines. Samples were loaded onto each lane of a 4-12% NuPAGE Bis-Tris gel and proteins were separated by electrophoresis. After transferring to nitrocellulose membrane, mPGES were detected by a specific anti-mPGES antibody. *Lane 1* and *7* shows a purified mPGES protein standard. *Lanes 2* to *6* are samples isolated from Flp-In 293 cells line that was co-transfected mPGES with COX-1, COX-1 *ins*580-598, COX-2 Y371F, COX-2 *del581-598* and Flag-tagged COX-2 respectively. *Lane 8* is a homogenate from cell transfected with a control plasmid. *Lanes 9* was sample isolated from Flp-In 293 cells line that was co-transfected mPGES with Flag-tagged COX-2.

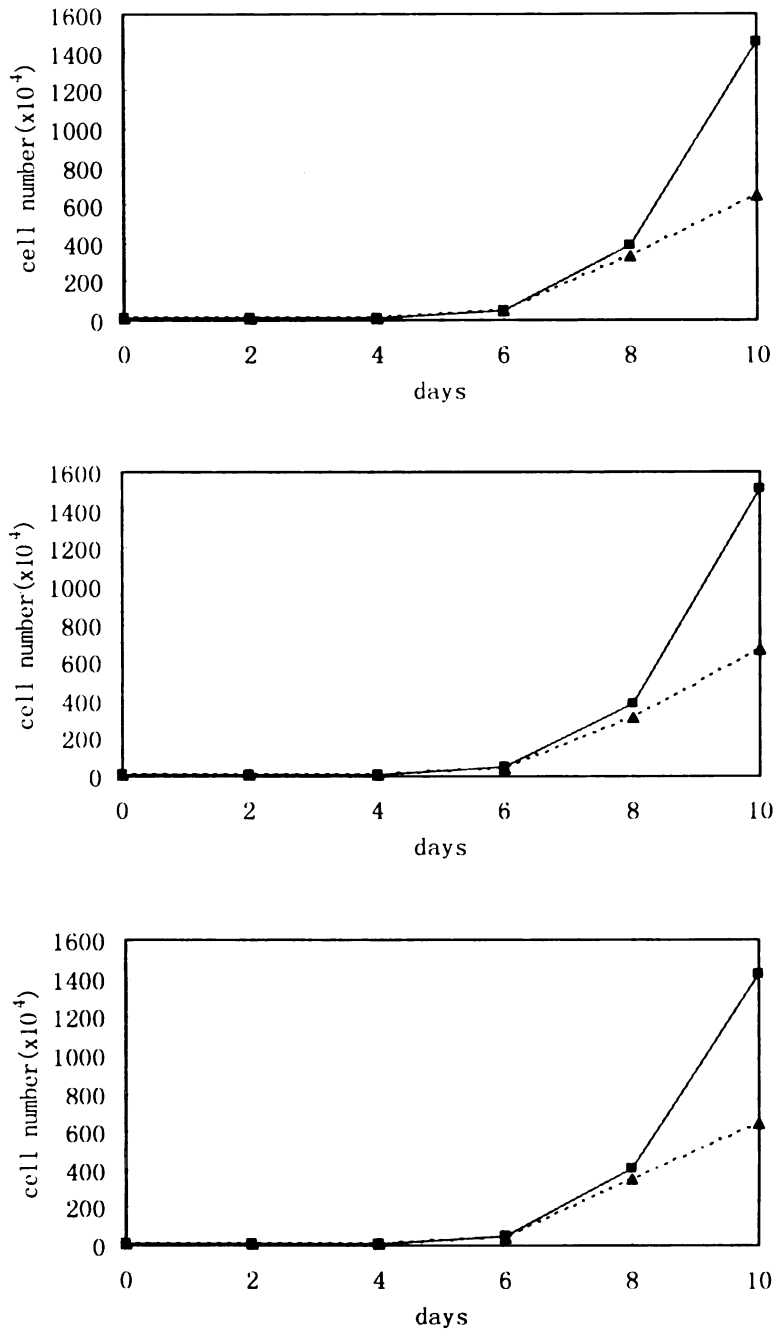


Figure 18((a)-1-3). Growth curve for 293 T-Rex cell lines expressing Flag-tagged COX-2 and mPGES (Solid line is tetracycline induced cells and dash line is cell control. 1×10^{4} or 5×10^{4} cells were inoculated at day 0). Results are shown in three independent experiments.

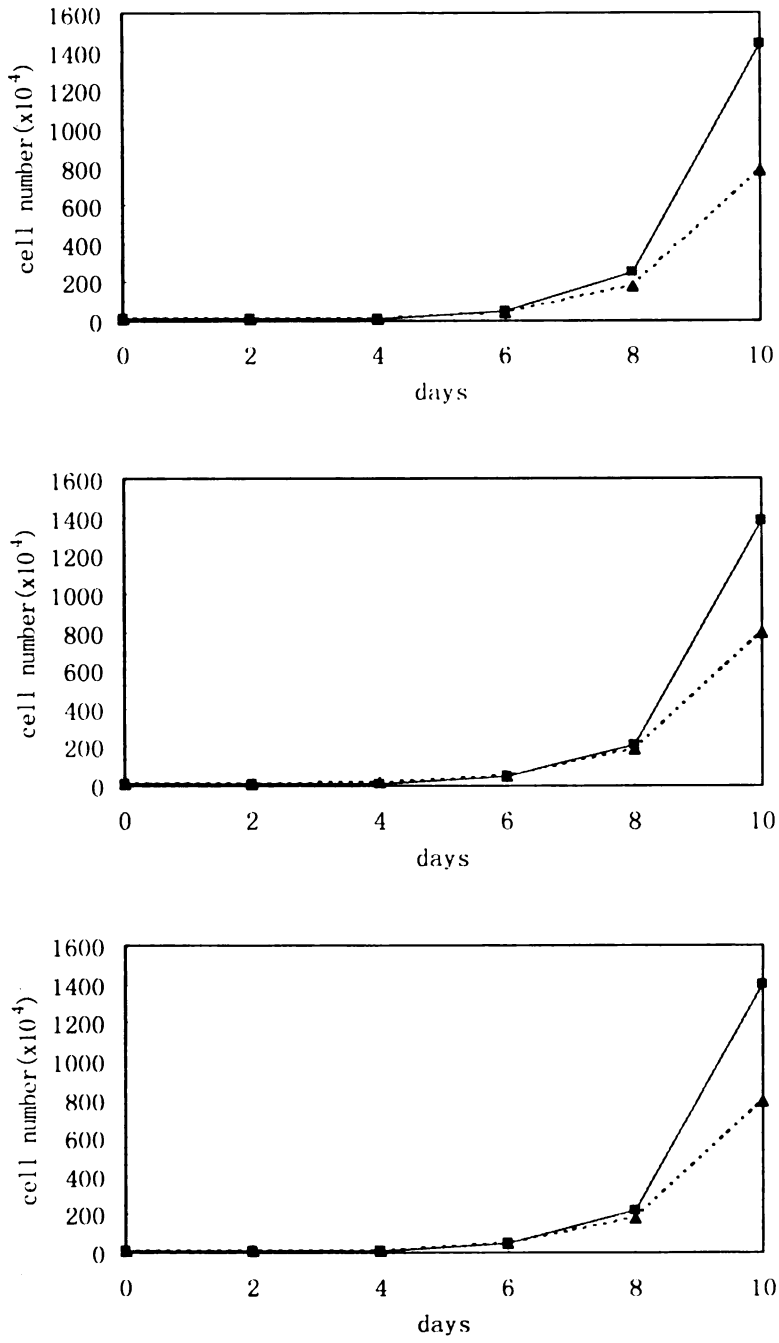


Figure 18((b)-1-3). Growth curve for 293 T-Rex cell lines expressing Flag-tagged COX-2 *del*581-598 and mPGES (Solid line is tetracycline induced cells and dash line is cell control. 1×10^{4} or 5×10^{4} cells were inoculated at day 0). Results are shown in three independent experiments.

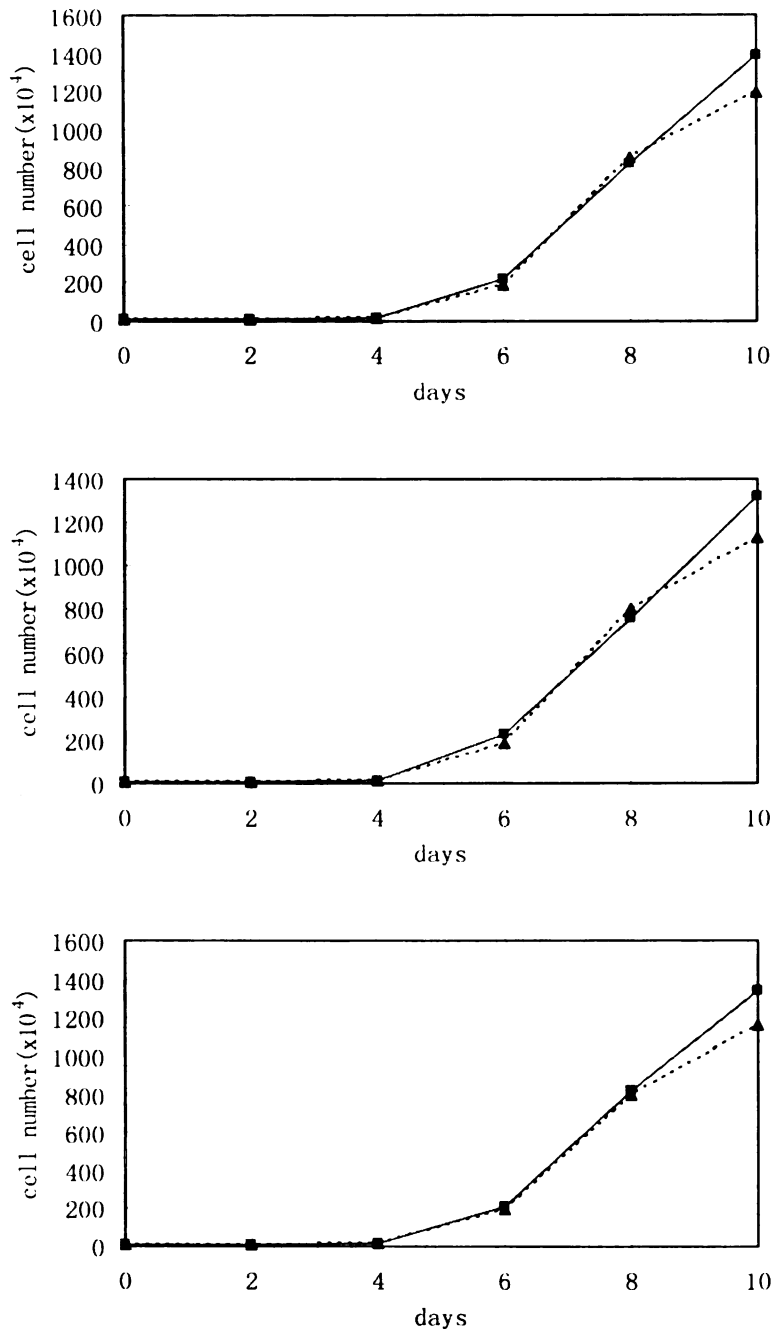


Figure 18((c)-1-3). Growth curve for 293 T-Rex cell lines expressing COX-1 *ins*580-598 and mPGES (Solid line is tetracycline induced cells and dash line is cell control. 1×10^{4} or 5×10^{4} cells were inoculated at day 0). Results are shown in three independent experiments.

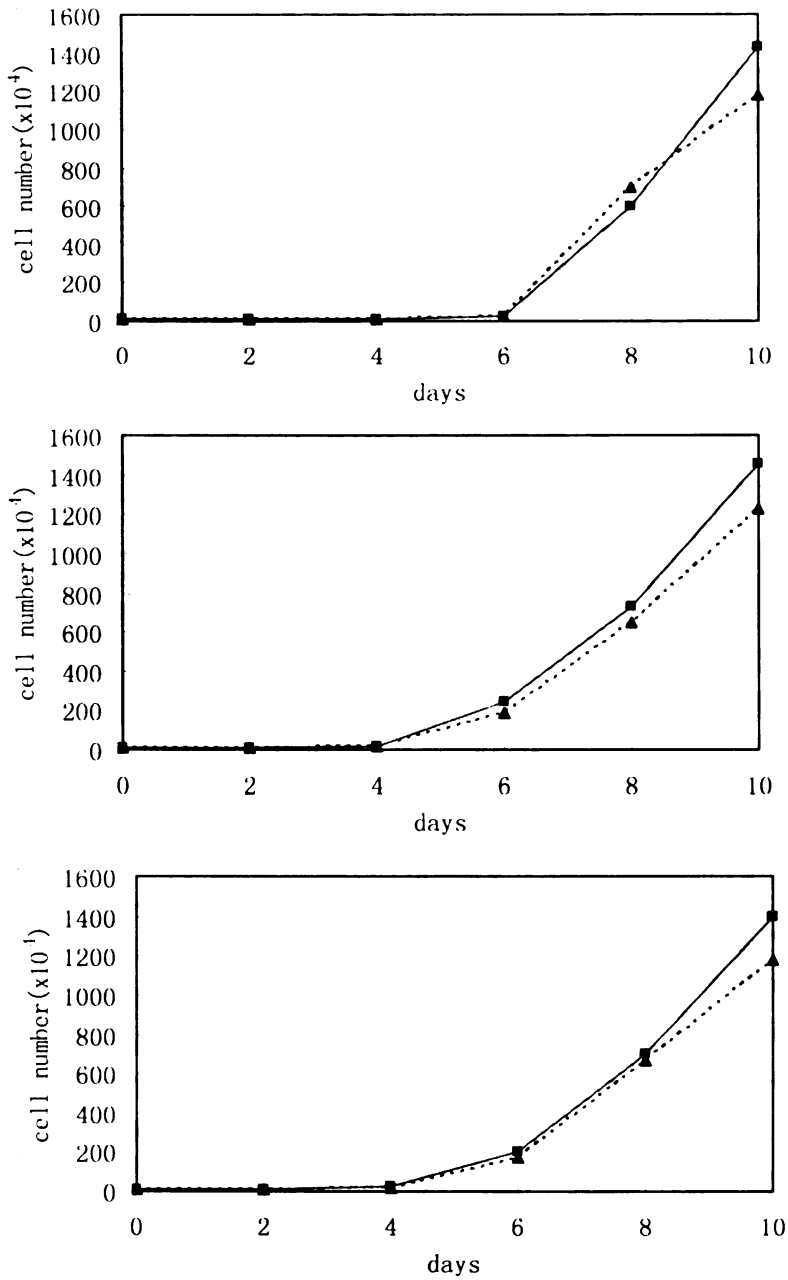


Figure 18((d)-1-3). Growth curve for 293 T-Rex cell lines expressing COX-1 and mPGES (Solid line is tetracycline induced cells and dash line is cell control. 1×10^{4} or 5×10^{4} cells were inoculated at day 0). Results are shown in three independent experiments.

Cell lines	COX Alone		COX + mPGES	
	Colonies	SIZE (mm)	Colonies	SIZE (mm)
No tetracycline Flag-tagged COX-2 cells only control	44	0.35	65	0.35
Tetracycline induced Flag-tagged COX-2 expressing cells	50	0.46	70	0.35
No tetracycline Flag-tagged COX-2 *del*581-598 cells only control	40	0.28	52	0.42
Tetracycline induced Flag-tagged COX-2 *del*581-598 expressing cells	38	0.35	48	0.37
No tetracycline COX-1 cells only control	40	0.26	38	0.33
Tetracycline induced COX-1 expressing cells	46	0.23	42	0.28

Table 5.Colony forming Agar assay for COX expressing transfectants. Stably transfected Flp-In 293 cells line expressing different COX mutants and the prostaglandin E synthase were tested for their ability to grow in the soft agar. Each experiment was repeated three times and similar results were obtained.

50μm cycloheximide for 0, 2, 4, 6, 8, 12, 24 hrs before harvesting. Expression of COX was determined by Western blotting and expression levels were quantitated by analyzing the band densities using Scion Image software (Fig 20(a-f)). $T_{1/2}$ was calculated by integrated first-order velocity equation; $2.3\log [S]_0/[S] = kt$ (Table 6).

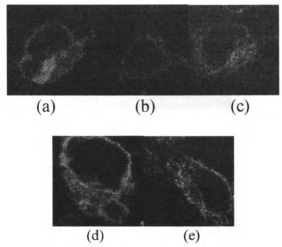
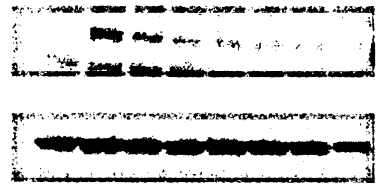


Figure 19(a-e). Immunocytochemistry of Flp-In 293 cells line stably transfected with Flag-tagged COX-2(a), COX-2 *del*581-598 (b), COX-2 Y371F(c), COX-1(d), COX-1 *ins*580-598 (e). Anti-FLAG antibody was used as the primary antibody and FITC-conjugated goat anti-mouse IgG was used as the secondary antibody. Stained cells were then examined under the microscope.

Three experiments were conducted, similar results were obtained and average $T_{1/2}$ was calculated.

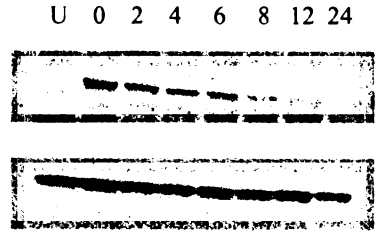
The $T_{1/2}$ for the COX-1 and COX-1 *ins*580-598 were both over 24 hr. COX-2 N580A, COX-2 *del*581-598, and COX-2 Y371F (the inactive COX-2 enzyme) had the similar $T_{1/2}$ compared to Flag-tagged COX-2. Since the entire turnover rates of COX-1 and COX-2 mutants were similar to each other respectively, this indicated that the activity and the 18 amino acids may not be important in the degradation process.

Determination turnover rates of untagged COX-2, COX-2 *del*581-598, COX-2 N580A, COX-1 and COX-1 *ins*580-598. Results in Smith's laboratory indicated (Uri' thesis) that the FLAG tag at N-terminus affected the turnover rate of COX enzymes, so the half-lives of untagged proteins were next determined. Stably-transfected HEK



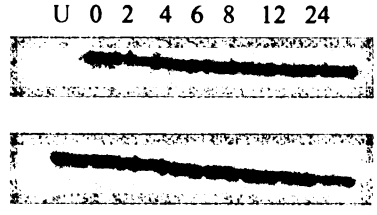
$T_{1/2}$=2.9h

Fig 20(a). $T_{1/2}$ for Flag-tagged COX-2



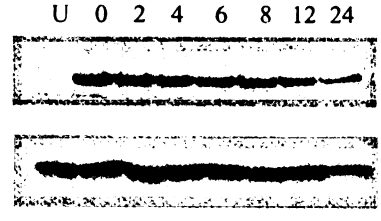
$T_{1/2}$=4.5h

Fig 20(b). $T_{1/2}$ for Flag-tagged COX-2 *del*581-598



$T_{1/2}$>24h

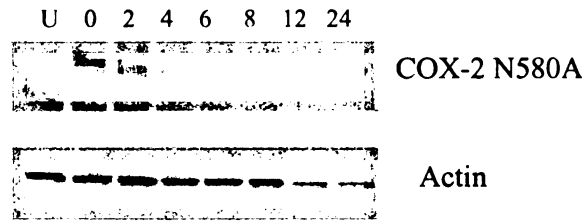
Fig 20(c). $T_{1/2}$ for Flag-tagged COX-1



$T_{1/2}$>24h

Fig 20(d). $T_{1/2}$ for Flag-tagged COX-1 *ins*580-598

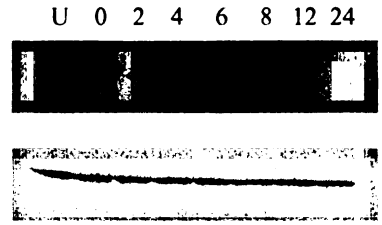
Fig 20 (cont'd)



$T_{1/2}$=2.3h

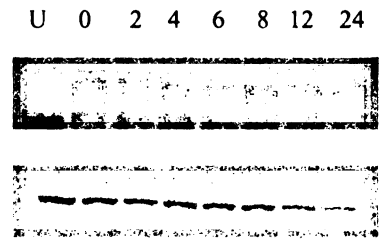
Fig 20(e). $T_{1/2}$ for Flag-tagged COX-2 N580A

Figure 20(a-e). Determination of $T_{1/2}$ for different COX mutants. Samples were loaded onto each lane of a 4-12% NuPAGE Bis-Tris gel and proteins were separated by electrophoresis. After transferring to nitrocellulose membrane, COX isoforms were detected by a specific anti-flag antibody or an anti-COX antibody. From left to right is cell only control and 0, 2, 4, 6, 8, 12, 24 hr (after CHX treatment) cell samples from Flp-In 293 cells line stably transfected with Flag-tagged COX-2(a), COX-2 *del*581-598(b), COX-1(c), COX-1 *ins*580-598(d), COX-2 N580A(e) respectively. Actin control was also included. Each experiment was repeated three times and similar results were obtained, average $T_{1/2}$ was calculated and presented.



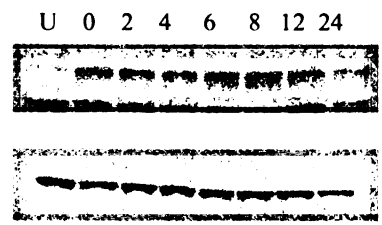
$T_{1/2}$=6.5h

Fig 21(a). $T_{1/2}$ for COX-2



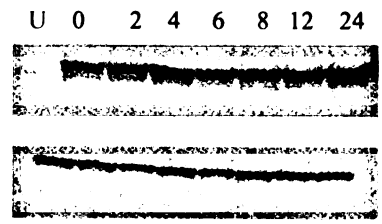
$T_{1/2}$>24h

Fig 21(b). $T_{1/2}$ for COX-2 *del*581-598



$T_{1/2}$>24h

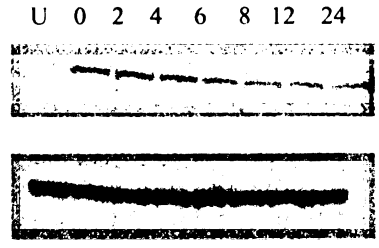
Fig 21(c). $T_{1/2}$ for COX-2 N580A



$T_{1/2}$>24h

Fig 21(d). $T_{1/2}$ for COX-1

Fig 21 (cont'd)



$T_{1/2}$=21h

Fig 21(e). $T_{1/2}$ for COX-1 *ins*580-598

Figure 21(a-e). Determination of $T_{1/2}$ for different COX mutants. Samples were loaded onto each lane of a 4-12% NuPAGE Bis-Tris gel and proteins were separated by electrophoresis. After transferring to nitrocellulose membrane, COX isoforms were detected by a specific anti-flag antibody or an anti-COX antibody. From left to right is cell only control and 0, 2, 4, 6, 8, 12, 24 hr (after CHX treatment) cell samples from Flp-In 293 cells line stably transfected with COX-2(a), COX-2 N580A(b), COX-2 *del*581-598(c), COX-1(d), COX-1 *ins*580-598(e) respectively. Actin control was also included. Each experiment was repeated three times and similar results were obtained, average $T_{1/2}$ was calculated and presented.

293 T-Rex cells were cultured and induced for cyclooxygenase expression by adding 2μg/ml tetracycline for 24 hrs. The cells were then incubated with the medium containing 50μm cycloheximide for 0, 2, 4, 6, 8, 12, 24 hrs before harvesting. Expression of COX was determined by Western blotting and expression levels were quantitated by analyzing the band densities using Scion Image software (Fig 21(a-f)). $T_{1/2}$ was calculated by integrated first-order velocity equation; $2.3\log [S]_0/[S] = kt$ (Table 6). Three experiments were conducted with similar results and average $T_{1/2}$ was calculated.

The tagged oCOX and native oCOX-1 had similar $T_{1/2}$. Untagged COX-1 *ins*580-598 had a slightly shorter $T_{1/2}$ than the tagged protein (21hr verses >24hr). The half life for $T_{1/2}$COX-2 was similar to the Flag-tagged COX-2 (2.9hr verses 6.5hr).

Surprisingly, COX-2 *del*581-598 and COX-2 N580A had a much longer $T_{1/2}$ (>24hr) compared to their tagged protein (24hr verses 4.5 and 2.3hr). Overall COX-1 containing 581-598 amino acids had a shorter $T_{1/2}$ than COX-1 and $T_{1/2}$ of COX-2 *del*581-598 was much longer than the COX-2. Adding the 18 amino acids increased the turnover rate of COX-1, and deleting the 18 amino acids decreased the turnover rate of COX-2, suggesting that the 18-amino acids insert promotes degradation. Furthermore COX-2 N580A had a much longer $T_{1/2}$ than COX-2 which suggests this C-terminal glycosylation site is also important in the turnover regulation of COX proteins.

Protein	$T_{1/2}$ (hr)	$T_{1/2}$ (hr) (Ave)
Flag-tagged COX-1 *ins*580-598	>24.0, >24.0, >24.0	>24.0
Flag-tagged COX-1	>24.0, >24.0, >24.0	>24.0
Flag-tagged COX-2	3.2, 2.5, 3.0	2.9
Flag-tagged COX-2 *del*580-598	4.0, 4.5, 5.0	4.5
Flag-tagged COX-2 N580A	2.8, 1.9, 2.2	2.3
COX-1 *ins*580-598	21.2,21.9, 19.9	21.0
COX-1	>24.0, >24.0, >24.0	>24.0
COX-2	7.5, 6.4, 5.6	6.5
COX-2 *del*580-598	>24.0, >24.0, >24.0	>24.0
COX-2 N580A	>24.0, >24.0, >24.0	>24.0

Table 6. Half-life of COX proteins. Stably transfected Flp-In 293 cells line expressing different COX mutants were used to test protein turnover rate. Each experiment was repeated three times and average $T_{1/2}$ was calculated and presented.

Discussion

To determine what structure or catalytic features of COX-2 may prevent its expression, transfection experiments with different forms of COX protein were carried out and the relative efficiency of isolating clones expressing different mutants was determined. The results indicated that COX-2 had the lowest expression ratio while deletion of the 18-amino acid cassette or inactivation of the enzyme could increase protein expression ratio, indicating that both activity and the 18-amino acid cassette were important for the protein expression. The Flag-tagged COX-2 *del*581-598 had a longer half life ($T_{1/2}$=4.5h) compared to the native Flag-tagged COX-2 ($T_{1/2}$=2.9h), the small difference between protein stability could in part explain the difficult to isolating the native COX-2 expressing colony. Results in Smith's lab indicated that inactive COX-2 mutant resisted to the AA induced degradation of COX-2 which clarified why COX-2 Y371F had a higher ratio of expression compared to native COX-2 (Smith's lab, Uri's thesis).

Meanwhile NS398 was also able to increase the expression ratio of the native COX-2. The outcome was much more dramatic than the inactive mutant (COX-2 Y371F) since it increased the ratio from 27% to 92% compared to only 46%. These results indicated additional potential role of NS398 other than simply inhibiting COX-2. Results in Smith's lab showed that NS398 could inhibit the substrate induced degradation of COX-2 (Smith's lab, Uri's thesis). The possible mechanism is that after binding to the enzyme, the conformation could be altered so the NS398-COX-2 complex is resistant to any other modification.

Flag-tagged COX-1 *ins*580-598 had 100% expression in all the colonies, same as Flag-tagged COX-1. The reason that 18 amino acids insert had no influence could due to the fact that both protein had the similar half life ($T_{1/2}$>24h) because of the Flag

tag. To verify the role of 18 amino acids insert in this case, expression ratio of untagged COX-1 *ins*580-598 needs to be determined.

Evidence has indicated that NSAIDs can inhibit colon cancer cell growth by inhibiting COX-2 (140). To determine if the COX enzymes can stimulate cell growth and if the 18-amino acid insert has a role in the process, growth curve and colony forming agar assay were conducted. We were unable to detect any difference when cells were expressing COX proteins alone. When COX proteins were co-expressing with the prostaglandin E synthase, our data confirmed the results in Kudo's laboratory that the coupling happened between COX-2 and mPGES, not COX-1. This coupling led to the increasing PGE_2 production which in turn can stimulate cell growth. However both Flag-tagged COX-2 and COX-2 *del*581-598 expressing cells (with or without mPGES) had the same phenotype in both experiments indicating that the 18 amino acids itself may not be involved in cell growth.

The role of 18-amino acid cassette in subcellular location has been studied by immunocytochemistry and no differences were observed for any of the COX proteins: similar staining of the perinuclear envelope and ER membrane were observed for all COX isoforms. The results indicated that 18-amino acid cassette does not affect the subcellular localization of the protein

When half-life study of COX protein was first studied, the Flag-tagged COX proteins were used and the entire turnover rates of COX-1 and COX-2 mutants were similar to each other respectively, suggesting that the 18 amino acids may not be important in the degradation process. However the results from Smith's laboratory indicated that the N-terminal tag (His tag or Flag tag) may significantly affect the turnover rate of COX enzymes (unpublished data). Since either His or Flag tag was just a small peptide, how they had such a striking effect on the protein degradation

remains mysterious. When focusing on the untagged COX protein, results in Smith's were similar to ours. COX-1 containing 581-598 amino acids had a shorter $T_{1/2}$ than COX-1 and $T_{1/2}$ of COX-2 *del*581-598 was much longer than the COX-2. Since adding the 18 amino acids could increase the turnover rate of COX-1 and deleting the 18 amino acids could decrease the turnover rate of COX-2, it indicated the role of 18 amino acids in the degradation pathway. Furthermore COX-2 N580A had a much longer $T_{1/2}$ than COX-2 which means this C-terminal glycosylation site was also important in the turnover regulation of COX proteins. Subtle difference in the catalytic activities of COX-2, and its different stability and expression profile may explain the different biological functions between the two isozymes.

In summary, our results demonstrated that 18-amino acid insert had no effect on cyclooxygenase activity, but reduced the number of colonies that could be detected that stably expressed COX-2. The 18 amino acid insert had no effect on COX-2 protein subcellular location and cell growth. The 19 amino acids at the C-terminus of COX-2 were important in the turnover regulation of COX proteins. The Flag-tag at the N-terminal of COX protein can significantly affect the protein turnover rate by unknown reasons. Whether the Flag-tag also affect other features of COX proteins are not clear yet. To solve this problem, untagged protein or different Tags should be used instead of Flag-tag.

CONCLUSION

My research focused on identifying putative protein partners for cyclooxygenase proteins by mass spectrometry analysis and determining the role of 18-amino acid cassette on cyclooxygenase cell biology.

To fulfill the first goal, different COX proteins (Flag-tagged native COX-2, COX-2 *del*581-598, COX-1 and COX-1 *ins*580-598) were expressed and purified for the proteomic analysis to identify the potential partners. Only two proteins were reproducibly identified, FAM44A (GI/Q8NFC6) and Heat shock-induced protein (GI/188492). FAM44A was identified in 5 out of the 7 experiments in protein complexes with COX-2 purified from T-Rex 293 cells, and 1 in 2 of the experiments where COX-2 was expressed transiently in Freestyle 293 cells. FAM44A was also identified in protein complexes with COX-1 *ins*580-598 but never in the uninduced 293 cells, or in protein complexes with COX-1 or COX-2 *del*581-598, suggesting association requiring a specific interaction with the 18-amino acid cassette of COX-2. FAM44A has been reported as a 330KDa protein. Its cDNA was isolated from human chromosomes 4 (114). FAM44A protein has AT-hook DNA binding domain which is prevalent in many eukaryotic nuclear proteins (116) and FAM44A can be phosporylated during DNA damage. Thus the interaction between COX-2 and FAM44A could be an alternative way to affect cell cycle. Another possibility is that COX-2 may interact with FAM44A for gene transcription regulation. FAM44A may serve as a chaperon for the COX-2-derived prostanoids to import into the nuclear to bind its target receptors for different gene expression regulation. The heat shock-induced protein was another protein that was identified in both T-Rex and Freestyle systems associating with COX-2. It was identified 1 out of the 7 experiments in

protein complexes with COX-2 purified from T-Rex 293 cells, and 1 in 2 experiments where COX-2 was expressed transiently in Freestyle 293 cells. Since heat shock-induced protein was not present in complexes with the COX-1 *ins*580-598 or the COX-2 *del*581-598, the interaction may require COX-2 protein domains in addition to the 18-amino acid cassette of COX-2. Heat shock-induced protein may aid in the processing of COX-2 or be involved in its degradation. Recent work has proven that this protein can inhibit LPS-induced NF-κB signaling cascade activation and subsequently decrease COX-2 expression (140), thus the interaction between COX-2 and heat shock-induced protein may also affect the expression of COX-2. Other proteins like BiP and KIAA0139 were only identified once in all 9 experiments indicating they could be an artifact due to over-expression of COX-2, or an anomaly of the isolation in a specific experiment.

No unique proteins were ever identified for COX-2 *del*581-59in the T-Rex cells. The identification of proteins that specifically co-purified with COX-2 and not with COX-2 *del*581-598 suggests that the 18 amino acids may be important in protein-protein interaction.

No interesting proteins were ever identified for COX-1 in the T-Rex cells. Also no protein partners were identified in common for COX-1 and COX-2. Since COX-1 and COX-2 have very different biological functions, it is reasonable to assume that the specific interaction identified in COX-2 may cause different functions between COX isozyme.

Among the proteins found in the COX-1 *ins*580-598 construct, FAM44A protein was the most probable protein partner candidate. As stated above, this protein was also co-purified with COX-2 complex and may association with COX-2 specifically via the

18-amino acid cassette. Although several other proteins were also identified, none of them were co-isolated for both COX-1 and COX-1 *ins*580-598. Thus, these proteins could just be the artifacts of over-expression.

To determine the role of 18-amino acid cassette on cyclooxygenase cell biology, the following aspects were checked: expression in 293 Flp-in cells, cell growth, subcellular localization and protein stability.

The ratio of expression results indicated that COX-2 had the lowest expression ratio while deletion of the 18-amino acid cassette or inactivation of the enzyme could increase protein expression ratio, indicating that both activity and the 18-amino acid cassette were important for the protein expression. That COX-2 activity has a negative effect on cell growth was also confirmed by addition of a COX-2 specific inhibitor NS398 during the transfection process

We were able to confirm the coupling between COX-2 and mPGES, not COX-1. However both Flag-tagged COX-2 and COX-2 *del*581-598 expressing cells had the same phenotype indicating that the 18 amino acids itself may not be involved in cell growth.

No differences were observed for any of the COX proteins subcellular localization: similar staining of the perinuclear envelope and ER membrane were observed for all COX isoforms. The results indicated that 18-amino acid cassette does not affect the subcellular localization of the protein

The results in our lab confirmed the insertion of the 18-amino acid cassette into COX-1 can destabilize the protein while deletion of the 18-amino acid cassette in COX-2 stabilized the protein, indicating the role of 18 amino acids in the degradation pathway.

Furthermore COX-2 N580A had a much longer $T_{1/2}$ than COX-2 which means this C-terminal glycosylation site was also important in the turnover regulation of COX proteins.

The explanation for why not many reliable candidates were identified could be due to the followings: the interactions are transient, or that the interaction of these proteins have low affinity and do not survive purification. Furthermore 293 cells do not normally express endogenous COX-2, and may not express protein partners for COX-2. Another problem we are having is the high background of non-specific (isolated and identified in both COX expressing and control samples) proteins. We assume that they could non-specifically bind to anti-flag agrose beads during purification. It is possible that when these proteins bind to the agrose beads it may affect even block the binding of potential protein partners. Besides the signals of these proteins are so intense, it may mask the signal from the real protein partners which is less redundant during the mass analysis, thus miss the identification of protein partner. To overcome these problems, first of all new cell should be used instead of the 293 cells. The cells should endogenously express COX isozymes to guarantee that potential protein partners exist. Secondly, new tag instead of Flag-tag should be used on COX protein for purification. When the protein complexes were purified with Flag-tag, the mass date contains high background because many non-specific proteins were attaching to the Flag agrose beads.

Another problem with the Flag-tag is that it significantly affected the turnover rate of COX enzymes (Smith's lab, unpublished data). Since Flag-tag was just a small peptide, how it had such a striking effect on the protein degradation remains mysterious. We already proved that Flag-tag would not affect the catalytic activity of COX-2. Whether

the Flag-tag can affect other features of COX proteins is not clear yet. To solve this problem, untagged protein or different Tags should be used instead of Flag-tag.

APPENDIX

Table 7. Plasmids constructed by QuickChange™ site-directed mutagenesis

PARENT PLASMID	PRODUCT PLASMID	PRIMERS SEQUENCES
hCOX-2-pFastBac	Flag-tagged COX-2-pFastBac	5'GCGCTCAGCCATACAGCAAATTGCGAGAACTTA TACTTTCAGGGACCTTGCTGTTCCCACCCATGT 3' 5'ACATGGGTGGGAACAGCAAGGTCCCTGAAAGTA TAAGTTCTCGCAATTTGCTGTATGGCTGAGCGC 3'
Flag-tagged hCOX2-pFastBac	FLAG-TEV COX-2-pFastBac	5'AGCCATACAGCAAATGATTACAAAGACGATGAC GATAAGTGCGAGAACTTATACTTTCAG 3' 5'CTGAAAGTATAAGTTCTCGCACTTATCGTCATCGT CTTTGTAATCATTTGCTGTATGGCT 3'
FLAG-TEV-COX-2-pFastBac	FLAG-TEV-COX-2 *del*581-598-pFastBac	5'ACAGTCACCATCAATGAACGTTCGAC 3' 5'CTACAGTTCAGTACGTTCATTG 3'
His-tagged COX-1-pIND	Flag-tagged COX-1-pIND	5'CGGGGCGCCCCGGGCTTATCGTCATCGTCTTTGTA ATCGTCCGCTGAGAAGACG 3' 5'TCTTCTCAGCGGACGATTACAAAGACGATGACGATA AGCCCGGGGCGCCCGCGC3'

Table 7 (cont'd)

His-tagged *ins*580-598 COX-1	Flag-tagged *ins*580-598 COX-1	5'CGGGGCGCCCCGGGCTTATCGTCATCGTCTTTGTA ATCGTCCGCTGAGAAGACG 3' 5'TCTTCTCAGCGGACGATTACAAAGACGATGACGAT AAGCCCGGGGCGCCCGCGC3'
FLAG-TEV COX-2-pCDNA5	FLAG-TEV-N580A-hCOX-2-pCDNA5	5'CATTAAAACAGTCACCATCGCTGCAAGTTCTTCCCG CTCC 3' 5'GGAGCGGGAAGAACTTGCAGCGATGGTGACTGTTT TAAT 3'
FLAG-TEV COX-2-pCDNA5	FLAG-TEV-Y371F-hCOX-2-pCDNA5	5'TTAACACCCTCTTCCACTGGCATCCCCTTC 3' 5' GGGATGCCAGTGGAAGAGGGTGTTAAATTC 3'
FLAG-TEV COX-2-pCDNA5	hCOX-2-pCDNA5	5'CAGCCATACAGCAAATCCTTGCTGTTCCCACC 3' 5' GGTGGGAACAGCAAGGATTTGCTGTATGGCTG 3'
FLAG-TEV *del*581-598 COX-2-pCDNA5	COX-2 *del*581-598-pCDNA5	5'CAGCCATACAGCAAATCCTTGCTGTTCCCACC 3' 5' GGTGGGAACAGCAAGGATTTGCTGTATGGCTG 3'
FLAG-TEV N580A-hCOX-2-pCDNA5	N580A-hCOX-2-pCDNA5	5'CAGCCATACAGCAAATCCTTGCTGTTCCCACC 3' 5' GGTGGGAACAGCAAGGATTTGCTGTATGGCTG 3'

Table 7 (cont'd)

Flag-tagged COX-1-pCDNA5	COX-1-pCDNA5	5'TTCTCAGCGGACCCCGGGGCGC 3' 5'CGCCCCGGGGTCCGCTGAGAAG 3'
Flag-tagged *ins*580-598 COX-1-pCDNA5	*ins*580-598 COX-1-pCDNA5	5' TTCTCAGCGGACCCCGGGGCGC 3' 5'CGCCCCGGGGTCCGCTGAGAAG 3'

PARENT PLASMID	PRODUCT PLASMID	RESTRICTION SITE
Flag-tagged COX-2 pFastBac	Flag-tagged COX-2-pCDNA5	*Not I*
Flag-tagged COX-2 *del*581-598-pFastBac	COX-2 *del*581-598-pCDNA5	*Not I*
Flag-tagged COX-1-pIND	Flag-tagged COX-1-pCDNA5	*Hind III*
Flag-tagged COX-1 *ins*580-598-pIND	Flag-tagged COX-1 *ins*580-598-pIND -pCDNA5	*Hind III*
Flag-tagged COX-2-pCDNA5	Flag-tagged COX-2-pOSML	*Not I*

Table 8. Plasmids constructed by subcloning

PCR-mediated mutation-Quick change

PCR-mediated introduction of mutations, inserts or deletions used either: 200ng and 20ng template vector, 5µl 10 x PCR reaction buffer (Stratagene), 2µl 10mM dNTP, 1µl polymerase (Strategene), 125 ng each primer and water was added to a total volume of 50µl. PCR conditions were adjusted for each prime set. Starting conditions were: 95°C for 1 minute, and 20-40 cycles of 95°C for 30 second, 50°C for 1 minute, and 68°C for various minutes depending on the size of plasmid (2 min/kb). A final extension step was employed at 68°C for 10 minutes and the reaction was lowered to 4°C.

Linker-Restriction Site Insertion

To change a restriction site, a single complementary oligo containing the new restriction site and a complementary to restriction site were ligated into cognate restriction digested plasmid. The self complementary oligo was first dissolved into Tris-EDTA containing 100mM NaCl at a concentration of 1mg/ml. The oligo was then heated to 80°C of water and allowed to cool to room temperature to from a double strand dimer. The hybridized oligo was stored at -20 °C. A typical ligation included 1µl hybrid oligo (1µg/µl), 1µl 10x ligation buffer (New England), 1µl plasmid (100ng/µl) digested at complementary site, 1µl T_4 ligase (New England) and H_2O to 10µl. The reaction was incubated at room temperature overnight, transformed the next day and analyzed for new restriction site by digestion of mini-prep samples of recombinant plasmids.

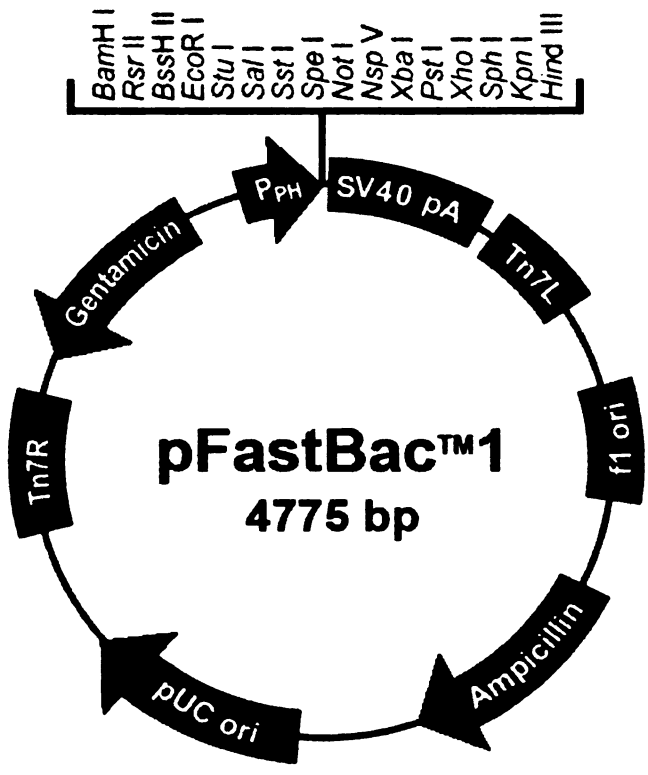


Figure 22. Map of COX-2 cloned into pFastBac (Invitrogen) at *Not I* site
(http://invitrogen.com/content/sfs/vectors/pfastbac1_map.pdf)

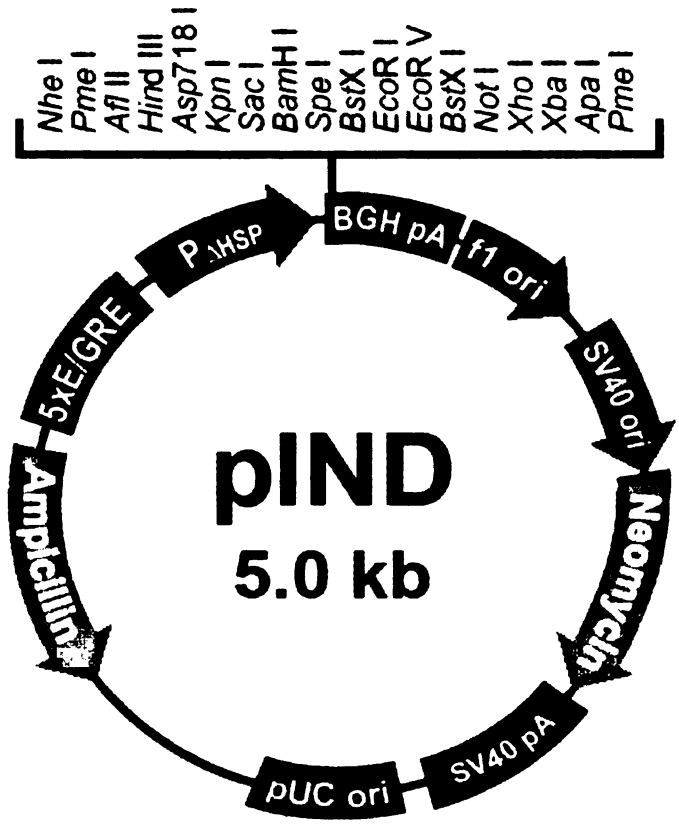


Figure 23. Map of His tagged-COX-1 cloned into pIND (Invitrogen) at *Hind III* site (http://invitrogen.com/content/sfs/vectors/pind_map.pdf)

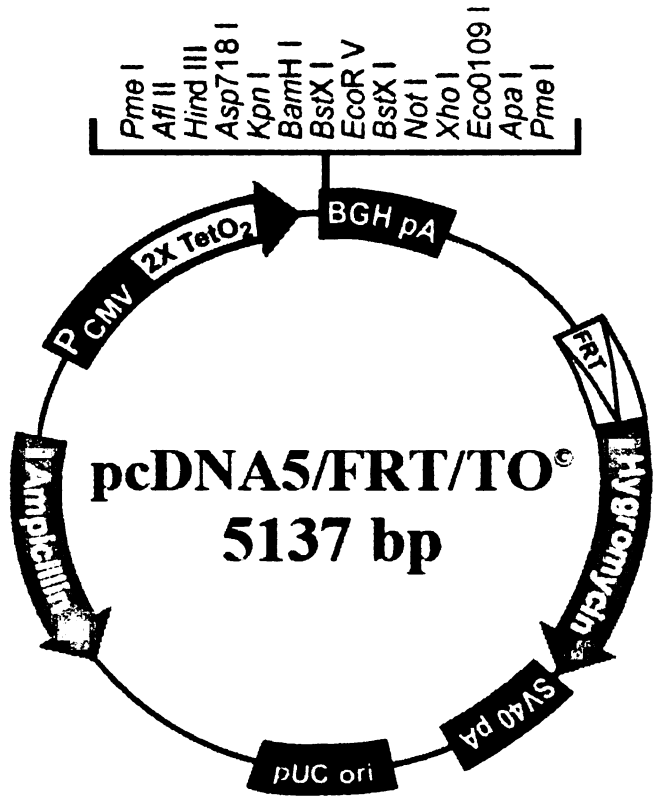


Figure 24. Map of Flag-tagged COX-2 and Flag-tagged COX-2 *del*581-598 subcloned into pcDNA5/FRT/TO (Invitrogen) vector from pFastbac at *Not I* site. Flag-tagged COX-1 and Flag-tagged COX-1 *ins*580-598 subcloned into pcDNA5/FRT/TO vector from pIND at *Hind III* site.

(http://invitrogen.com/content/sfs/vectors/pcdna5frtto_map.pdf)

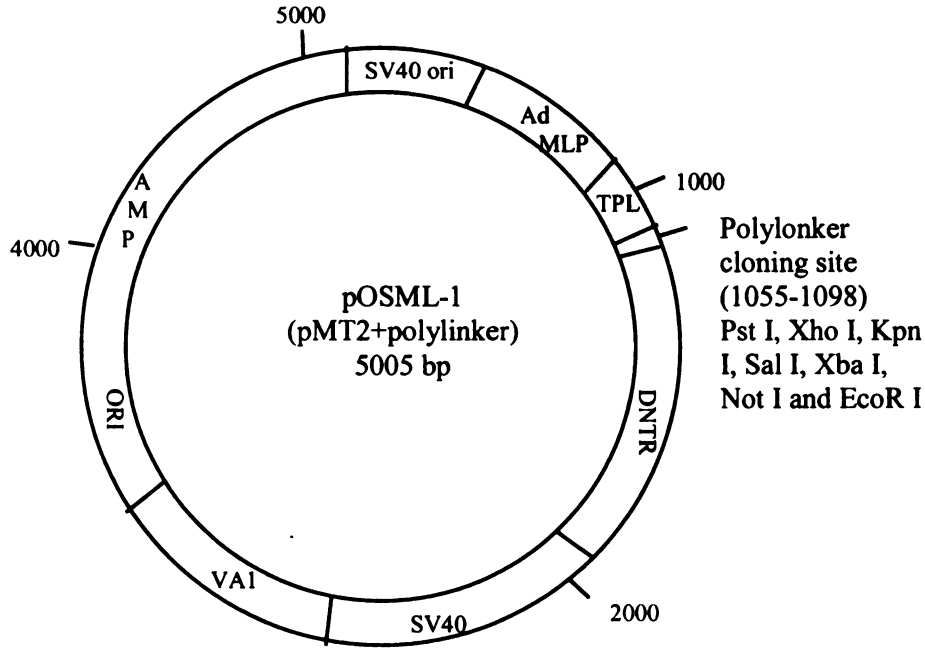


Figure 25. Map of Flag-tagged COX-2 subcloned into pOSML vector from pcDNA5/FRT/TO at *Not I* site.

(http://invitrogen.com/content/sfs/vectors/pcdna5frtto_map.pdf)

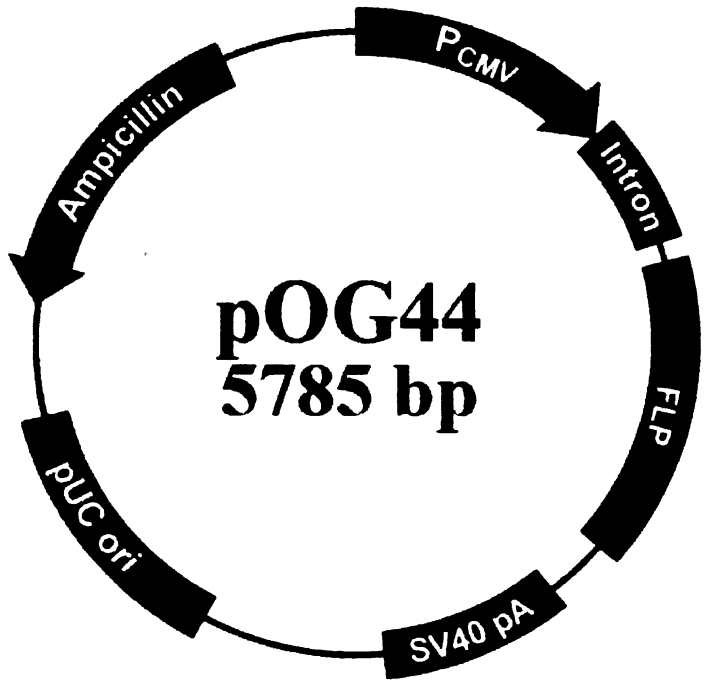


Figure 26. Map of pOG44 vector expressing Flp recombinase
(https://www.invitrogen.com/content/sfs/vectors/pog44_map.pdf)

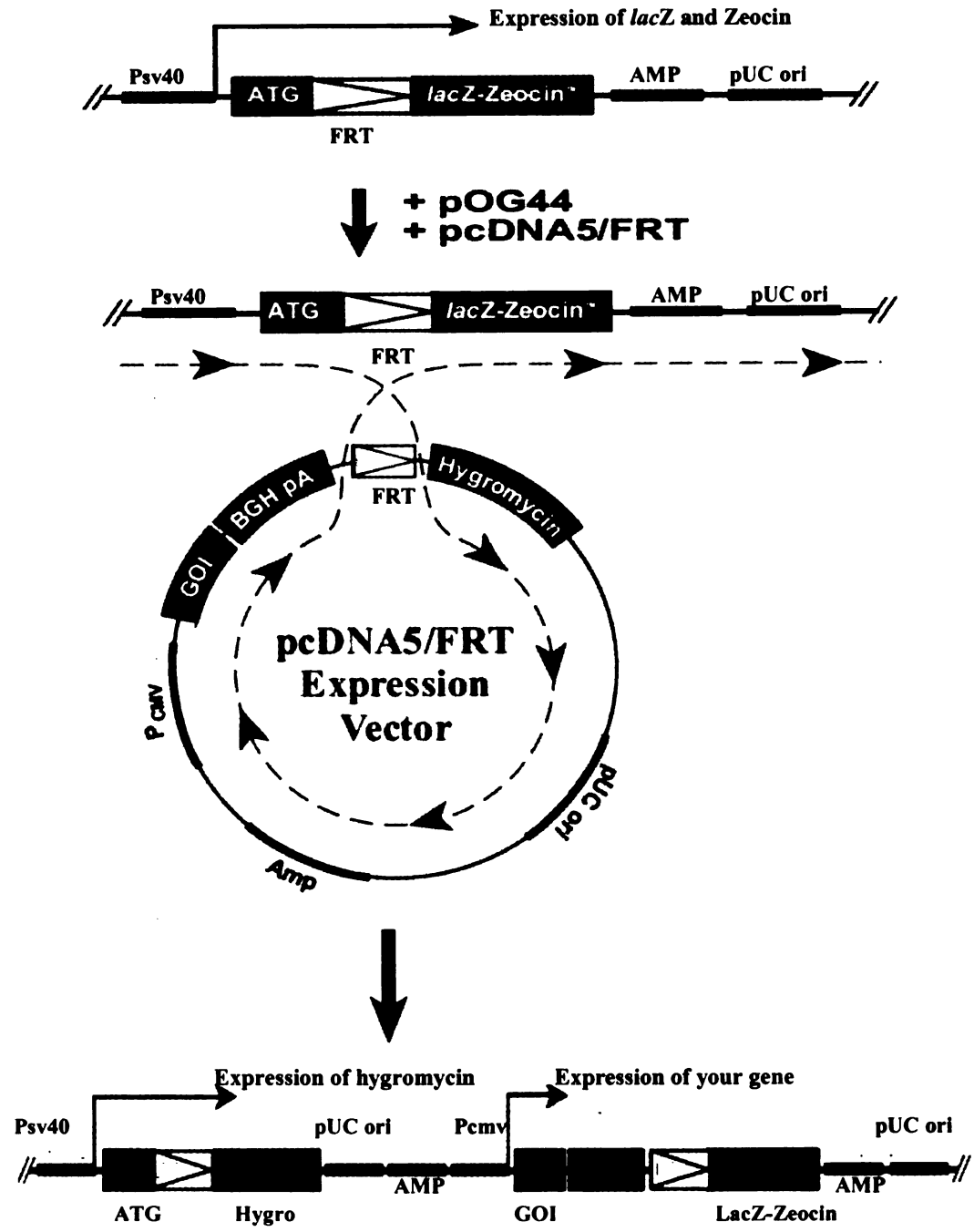


Figure 27. Flp-In-T-Rex Tetracycline Inducible System. The Flp-In T-Rex system exhibit tetracycline-inducible gene expression after specific integration of the transfected gene into the genome via Flp recombinase-mediated DNA recombination.
(www.invitrogen.com/content/sfs/manuals/flpinsystem_man.pdf).

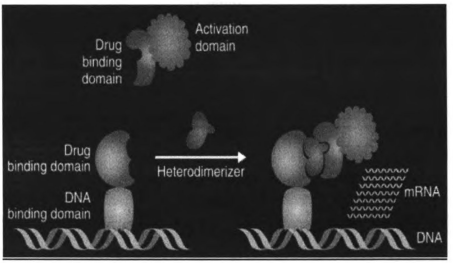


Figure 28(a). AP inducible expressing system. The transcription of a target gene under the control of a small molecule "dimerizer". (http://www.ariad.com/pdf/Reg_Tx-Retrovirus.pdf#search=%22AP%20inducible%20expression%20system%20Ariad%20Pharmaceuticals%20%22).

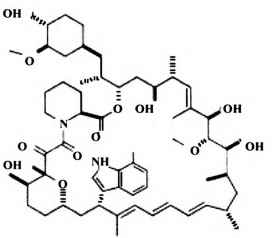


Figure 28(b). AP21967. It is a chemically modified derivative of rapamycin that can be used to induce heterodimerization of FKBP and FRB_{T2098L} -containing fusion proteins.

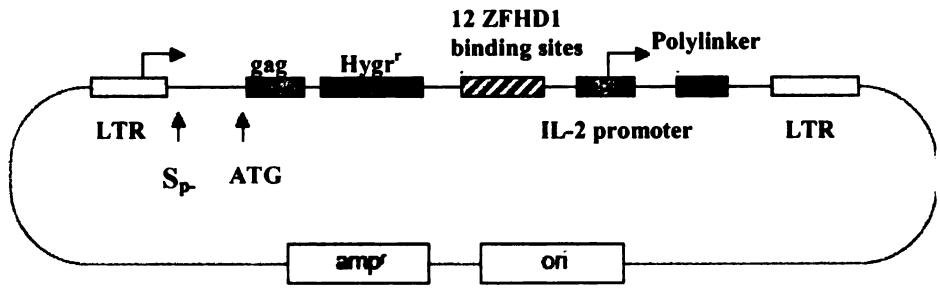


Figure 28(c). Target gene vector pLH-Z_{12}I-PL. Vector contains 12 ZFHD1 binding sites and a minimal human interleukin-2 gene promoter (Z_{12}I), upstream of a polylinker (PL) inserted downstream of an LTR-driven hygro resistance gene (Hygro^r). Insertion of the gene of interest into the polylinker places its expression under control of the dimerizer-regulated transcription factors.

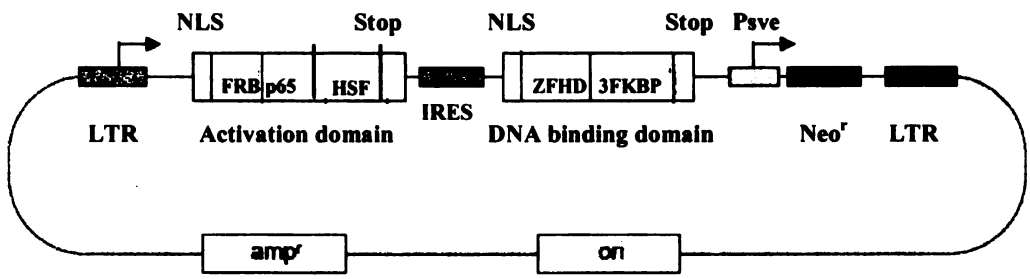


Figure 28(d). Transcription factor vector pL_2N_2-R_HS3H/ZF3. An activation domain fusion (RHS3H) which contains the FRB fragment of human FRAP (RH), fused to a highly potent chimeric activation domain called S3H. S3H consists of amino acids 281 to 551 from the p65 subunit of human NFκB (S3) and amino acids 406-530 from human heat shock factor 1(H). The FRB domain consists of amino acids 2021-2113 of FRAP, in which the threonine at amino acid 2098 is mutated to leucine. This mutation allows the protein to bind to rapamycin analogs (e.g. AP21967) which no longer bind appreciably to endogenous FRAP. A DNA binding domain fusion (ZF3) which consists of the ZFHD1 DNA binding domain (Z) and three tandemly repeated copies of human FKBP12 (F3).

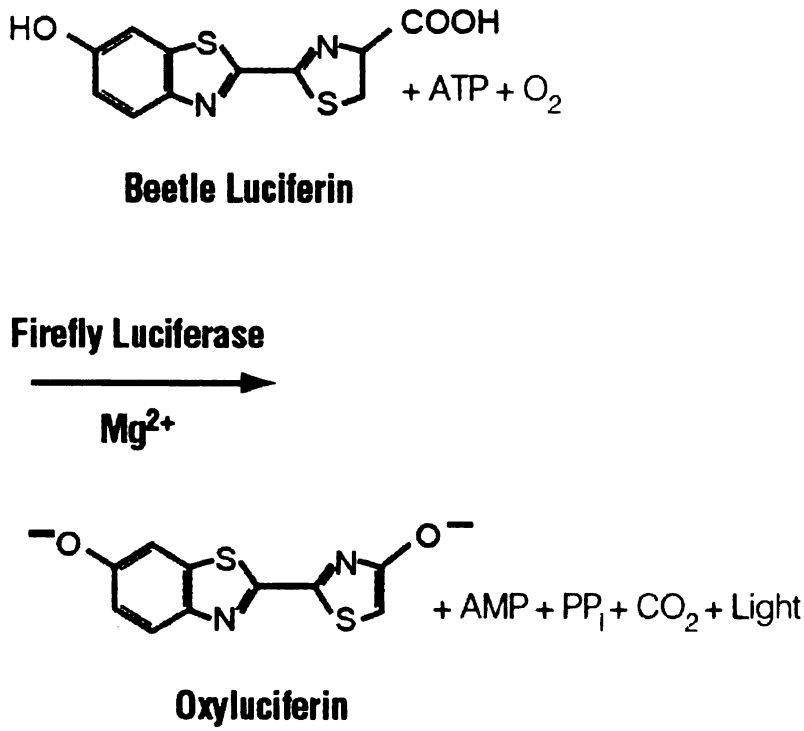


Figure 29. Bioluminescent reaction catalyzed by firefly luciferase.
(http://www.promega.com/tbs/tb281/tb281.pdf)

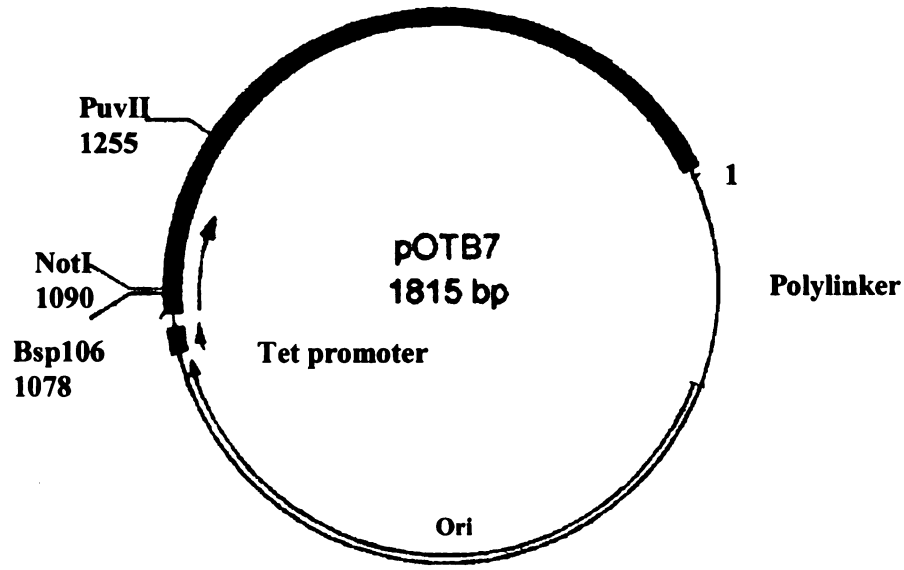


Figure 30. Map of vector pOTB7 that was source of mPGES (Invitrogen). cDNA made by oligo-dT primimg. Directionally cloned into *EcoR I/Xho I* sites using the following 5' adaptor: GGCACGAG (G) (www.rzpd.de/info/vectors/pOTB7_pic.shtml)

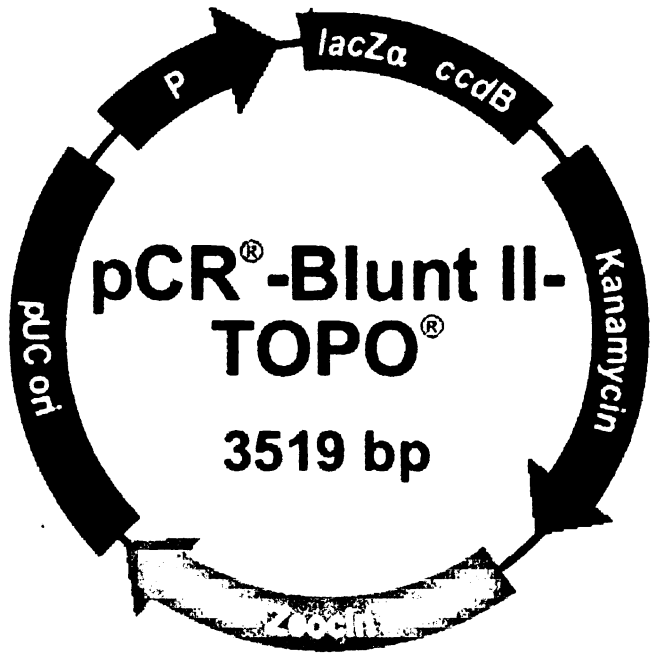


Figure 31. Map of mPGES cloned into Blunt TOPO II after PCR.
(http://www.invitrogen.com/content/sfs/manuals/zeroblunttopo_man.pdf)

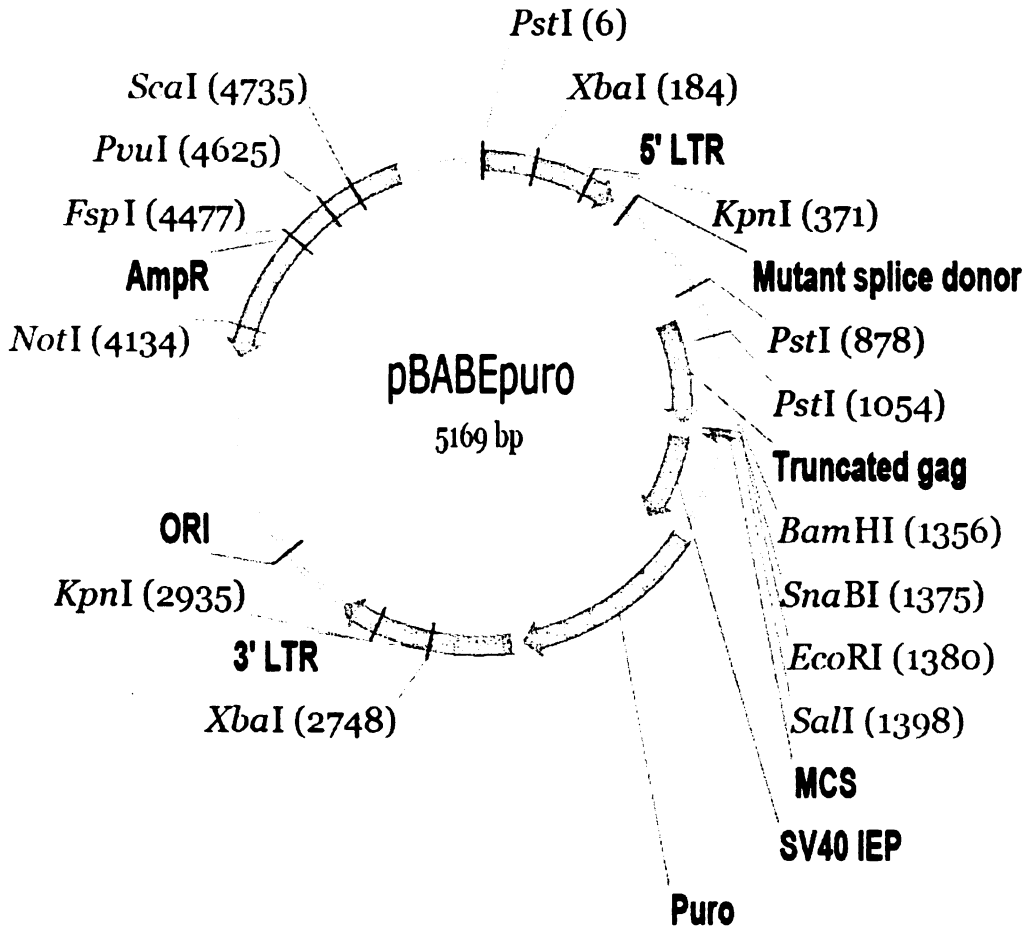


Figure 32. Map of mPGES cloned into pBABE-puro vector at *EcoR* I site.

BIBLIOGRAPHY

1. Smith WL, DeWitt DL and Garavito RM 2000 *Annu Rev Biochem* **69**:145-82

2. Castellone MD, Teramoto H, Williams BO, Druey KM and Gutkind JS 2005 *Science* **310**:1504

3. Kao HW, Hyman PE, Finn SE and Snape WJ Jr 1988 *Am J Physiol Gastrointest Liver Physiol* **255**:G807-G812,

4. Raud J, Dahlén SE, Sydbom A, Lindbom L and Hedqvist P 1989 *Inflammation Research* **26**:42-44

5. Narumiya S, Sugimoto Y and Ushikubi F 1999 *Physiol Rev* **79**:1193-1226

6. Schaefer M, Hofmann T, Schultz G and Gudermann T 1998 *Proc Natl Acad Sci* **95**: 3008-13

7. Negishi M, Ito S and Hayaishi O 1989 *J. Biol. Chem* **264(7)**:3916-3923

8. Andres DA, Dickerson IM and Dixon JE 1990 *J. Biol. Chem* **265(11)**:5952-5955

9. Xie WL, Chipman JG, Robertson DL, Erikson RL and Simmons DL 1991 *Proc Natl Acad Sci* **88(7)**:2692-6

10. Kulmacz RJ 1998 *FEBS Lett* **430:**154–157

11. Smith WL and Langenbach R 2001 *J Clin Invest* **107**:1491–1495

12. Yamamoto K, Arakawa T, Ueda N and Yamamoto S 1995 *J Biol Chem* **270:**31315–20

13. Subbaramaiah K, Chung WJ, Michaluart P, Telang N and Tanabe T 1998 *J Biol Chem* **273:**21875–82

14. DeWitt DL 1999 *Mol Pharmacol* **4:**625–31

15. Landino LM, Crews BC, Gierse JK, Hauser SD and Marnett LJ. 1997 *J Biol Chem* **272:**21565–74

16. Dietz R, Nastainczyk W and Ruf HH 1988 *Eu J Biochem* **171:**321-28

17. Lambeir AM, Markey CM, Dunford HB and Marnett LJ 1985 *J Biol Chem* **260:** 14894–96

18. Lu G, Tsai AL, VanWart HE and Kulmacz RJ 1999 *J Biol Chem* **274:**16162–67

19. Karthein R, Dietz R, Nastainczyk W and Ruf HH 1988 *Eur J Biochem* **171:**313-20

20. Tsai A, Wu G, Palmer G, Bamba B and Koehn JA 1999 *J Biol Chem* **274:** 21695–700

21. Smith WL, Lands WEM. 1972. *Biochemistry*11:3276–82

22. Tsai A, Kulmacz RJ and Palmer G 1995 *J Biol Chem* **270:**10503–8

23. Wu G, Wei C, Kulmacz RJ, Osawa Y and Tsai AL 1999 *J Biol Chem* **274:**9231–37

24. Tsai A, Hsi LC, Kulmacz RJ, Palmer G and Smith WL 1994 *J Biol Chem* **269:**5085–91

25. Hsi LC, Hoganson CW, Babcock GT, Garavito RM and Smith WL 1995 *Biochem Biophys Res Commun* **207:**652–60

26. Shi W, Hoganson CW, Espe M, BenderCJ and Babcock GT 2000 *Biochemistry* **39(14):**4112-21

27. Koshkin V, Dunford HB 1999 *Biochim Biophys Acta* **1430:**341–48

28. Hemler ME, Lands WEM. 1980 *J Biol Chem* 255:6253–61

29. Picot D, Loll PJ, Garavito RM 1994 *Nature* **367**:243–49

30. Luong C 1996 *Nat Struct Biol* 3:927-933.

31. Kurubail R 1998 *Second International Workshop on Cox-2*

32. DeWitt DL 1999 *Molecular Pharmacology* **55:**625–631

33. Smith WL and Reike CJ *personal communication*

34. Greig GM, Francis DA, Falgueyret J-P, Ouellet M, Percival MD, Roy P, Bayly C, Mancini JA and O'Neill GP 1997 *Mol Pharmacol* **52:** 829-838

35. Gierse JK, McDonald JJ, Hauser SD, Rangwala SH, Koboldt CM and Seibert K 1996 *J Biol Chem* **271:**15810–15814

36. Guo Q, Wang LH, Ruan KH and Kulmacz RJ 1996 *J Biol Chem* **271:**19134–19139

37. Kurumbail RG, Stevens AM, Gierse JK, McDonald JJ, Stegeman RA, Pak JY, Gildehaus D, Miyashiro JM, Penning TD, Seibert K, Isakson PC and Stallings WC 1996 *Nature London* **384:**644-648.

38. Kraemer SA, Meade SA and DeWitt DL 1992 *Arch Biochem Biophys* **293:**391–400

39. Xu XM, Tang JL, Chen X, Wang LH and Wu KK 1997*J Biol Chem* **272**(11): 6943-50

40. Xie W and Herschman HR 1996 *J Biol Chem* **271:**31742–48

41. Subbaramaiah K, Chung WJ, Michaluart P, Telang N and Tanabe T 1998 *J Biol Chem* **273:**21875–82

42. Morris JK and Richards JS 1996 *J BiolChem* **271:**16633–43

43. Gietz RD, Schiestl RH, Willems AR and Woods RA 1995 *Yeast* **11:**355-60

44. Roshak AK, Jackson JR, McGough K, Chabot-Fletcher M and Mochan E 1996 *J Biol Chem* **271(49):**31496-501

45. Crofford LJ, Tan B, McCarthy CJ and Hla T 1997 *Arthritis Rheum* **40:**226–36

46. Miller C, Zhang MK, He YL, Zhao J and Pelletier JP 1998 *J Cell Biochem* **69:**392–413

47. Jobin C, Morteau O, Han DS, Balfour-Sartor R 1998 *Immunology* **95:**537–43

48. Schmedtje JF Jr, Ji YS, Liu WL, DuBois RN, Runge MS 1997 *J Biol Chem* **272:**601–8

49. Gallois C, Habib A, Tao J, Moulin S and Maclouf J 1998 *J Biol Chem* **273(36):**23183–90

50. Ghosh S, May MJ and Kopp EB 1998 *Annu Rev Immunol* **16:**225–60

51. Zhang FX, Kirschning CJ, Mancinelli R, Xu XP and Jin Y 1999 *J Biol Chem* **274:**7611–14

52. Su B and Karin M 1996 *Curr Opin Immunol* **8:**402–11

53. Wallach D,Varfolomeev EE, Malinin NL,Goltsev YV and Kovalenko AV 1999 *Annu Rev Immunol* **17:**331–67

54. Xie W and Herschman HR 1995 *J Biol Chem* **270:**27622–28

55. Subbaramaiah K, Chung WJ and Dannenberg AJ 1998 *J Biol Chem* **273:**32943-49

56. Guan ZH, Buckman SY, Pentland AP, Templeton DJ and Morrison AR 1998 *J Biol Chem* **273:**12901–8

57. Scherle PA, Jones EA, Favata MF, Daulerio AJ and Covington MB 1998 *J Immunol* **161:**5681-86

58. Dean JL, Brook M, Clark AR and Saklatvala J 1999 *J Biol Chem* **274:**264–69

59. Adderley SR and Fitzgerald DJ 1999 *J BiolChem* **274:**5038–46

60. Ridley SH, Dean JL, Sarsfield SJ, BrookM and Clark AR 1998 *FEBS Lett* **439:**75–80

61. Guan Z, Baier LD and Morrison AR 1997 *J Biol Chem* **272:**8083–89

62. Guan ZH, Buckman SY, Miller BW, Springer LD and Morrison AR 1998 *J Biol Chem* **273**:28670–76

63. Sheng H, Williams CS, Shao J, Liang P and DuBois RN1998 *J Biol Chem* **273:**22120–27

64. Ridley SH, Sarsfield SJ, Lee JC, Bigg HF and Cawston TE 1997 *J Immunol* **158:**3165–73

65. Buckman SY, Gresham A, Hale P, Hruza G and Anast J 1998 *Carcinogenesis* **19:**723–29

66. Tanabe T and Tohnai N 2002 *Prostaglandins Other Lipid Mediat* **68:**95-114

67. Chen CY and Shyu AB 1994 *Molecular and cellular biology* **14(12)**:8471-8482

68. DeWitt DL and Meade EA 1993 *Arch Biochem Biophys* **306(1):**94-102

69. Chen CY and Shyu AB 1995 *Trends in biochemical sciences* **20(11)**:465470

70. Chen CY, Gherzi R, Ong SE, Chan EL, Raijmakers R, Pruijn GJ, Stoecklin G, Moroni C, Mann M and Karin M 2001*Cell* **107(4)**:451-464

71. Mukherjee D, Gao M, O'Connor JP, Raijmakers R, Pruijn G, Lutz CS and Wilusz J 2002 *J EMBO* **21(1-2)**:165-174

72. Stoecklin G, Mayo T and Anderson P 2006 *EMBO reports* **7(1)**:72-77

73. Dixon DA, Tolley ND, King PH, Nabors LB, McIntyre TM, Zimmerman GA and Prescott SM 2001 *J clinical investigation* **108(11)**:1657-1665

74. Mbonye UR, Wada M, Rieke CJ, Tang HY, DeWitt DL and Smith WL 2006 *J Biol Chem* **281**: 35770–35778

75. Figueiredo-Pereira ME, Li Z, Jansen M and Rockwell P 2002 *J Biol Chem* **277(28)**: 25283-25289

76. Sugimoto Y, Narumiya S, and Ichikawa A 2000 *Prog Lipid Res* **39:**289-314

77. Langenbach R 1995. *Cell* **83**:483-492

78. Lim H 1997 *Cell* **91**:197-208

79. Dinchuk JE 1995 *Nature* **378**:406-409

80. Komhoff M 2000 *Kidney Int.* **57**:414-422

81. Loftin CD 2001 *Proc Natl Acad Sci USA* **98**:1059-1064

82. Charkraboty I, Das SK, Wang J, Dey SK 1996 *J.Nol. Endocrinol* **16**:197-208.

83. Langenbach R, Loftin C, Lee CA, Tiano H 1999 *Biochemical Pharmacology* **58**: 1237-1246

84. Liu CH, Chang SH, Narko K, Trifan OC, Wu MT, Smith E, Haudenschild C, Lane TF, Hla T 2001 *J Biol Chem* **276**: 18563-18569

85. Giardiello FM, Offerhaus GJ, DuBios RN 1995 *Eur J Cancer* **7-8**: 1071-1076

86. Oshima M, Dinchunk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans JF, Taketo MM 1996 *Cell* **87**:4803-809

87. Chinery R, Robert JC, Ramona G, Kirkland SC, Stephanie CS, William EZ, John AO, and Morrow JD 1999 *Cancer Res* **59**: 2739-2746

88. Chinery R, Beauchamp RD, Shyr Y, Kirkland SC, Coffey RJ, and Morrow JD 1998 *Cancer Res* 58: 2323-2327

89. Castano E, Bartrons R, Gil J *Pharmacol Exp Ther.* 2000 **293**:509-13

90. Tsujii M and DuBois RN Cell 1995 **83(3):**493-501

91. Bakhle YS *Brit J Pharm* 2001 **134**:1137-1150

92. DuBois RN, Gupta R, Brockman J, Reddy BS, Krakow SL, Lazar MA 1998 *Carcinogenesis.* **19**:49-53

93. He TC, Chan TA, Vogelstein B and Kinzler KW 1999 *cell* **99**:335

94. Chulada PC, Thompson MB, Mahler JF, Doyle CM, Gaul BW, Lee C, Tiano HF, Morham SG, Smithies O and Langenbach R 2000*Cancer Res* **60:** 4705-8

95. Kitamura T, Kawamori T, Uchiya N, Itoh M, Noda T, Matsuura M, Sugimura T and Wakabayashi K 2002 *Carcinogenesis* **23:** 1463-6

96. Murakami M, Naraba H, Tanioka T, Semmyo N, Nakatani Y, Kojima F, Ikeda T, Fueki M, Ueno A, Oh S and Kudo I 2000 *J Biol Chem* **275:** 32783–32792

97. Jakobsson PJ, Thorén S, Morgenstern R and Samuelsson B 1999 *Proc Natl Acad Sci USA* **96(13):** 7220–7225.

98. Huang M, Stolina M, Sharma S, Mao JT, Zhu L, Miller PW, Wollman J, Herschman H and Dubinett SM 1998 *Cancer Res* **58(6):**1208-16

99. Yoshimatsu K, Golijanin D, Paty PB, Soslow RA, Jakobsson PJ, DeLellis RA, Subbaramaiah K and Dannenberg AJ 2001 *Clin Cancer Res* **7:**3971-3976

100. Abramovitz M, Wong E, Cox ME, Richardson CD, Li C and Vickers PJ 1993 *Eur J Biochem* **215(1):**105-11

101. Mancini JA, Abramovitz M, Cox ME, Wong E, Charleson S, Perrier H, Wang Z, Prasit P and Vickers PJ 1993 *FEBS Lett* **318(3):**277-81.

102. Turini ME and DuBois RN 2002 *Annu Rev Med* **53:**35.

103. Moncada S 1999 *J R Soc Med* 92:164.

104. Kim SF, Huri DA and Snyder SH 2005 *Science* **310(5756)**:1966-70

105. Neuss H, Huang XH, Hetfeld BKJ, Deva R, Henklein P, Nigam S, Mall JW, Schwenk W and Dubiel W 2007 *J Mol Med* **85:** 961-970

106. Simmons DL, Botting RM and Hla T 2004 *Pharmacol Rev* **56:**387–437

107. Tsai A, Palmer G, Xiao G, Swinney DC and Kulmacz RJ 1998 *J Biol Chem* **273:**3888-3894

108. Noden PA, Smith WL, DeWitt DL and Roux JF 1981*Biol Reprod* **24(5):**1042-7

109. Otto JC, DeWitt DL and Smith WL 1993 *J Biol Chem* **268:**18234–18242

110. Pollock R, Issner R, Zoller K, Natesan S, Rivera VM and Clackson T 2000 *Proc Natl Acad Sci USA* **97(24):**13221-6.

111. Zhang H, Wu W, Du Y, Santos SJ, Conrad SE, Watson JT, Grammatikakis N and Gallo KA 2004 *J Biol Chem* **279(19):**19457-63

112. Ting J, Lee AS 1988 *DNA* **7**: 275-286

113. Milner CM and Campbell RD 1990 *Immunogenetics* **32**: 242-251

114. Hillier LW, Graves TA, Fulton RS, Fulton LA, Pepin KH, Minx P, Wagner McPherson C, Layman D, Wylie K, Sekhon M, Becker MC, Fewell GA, Delehaunty KD, Miner TL, Nash WE, Kremitzki C, Oddy L, Du H, Sun H, Bradshaw-Cordum H, Ali J, Carter J, Cordes M, Harris A, Isak A, van Brunt A, Nguyen C, Du F, Courtney L, Kalicki J, Ozersky P, Abbott S, Armstrong J, Belter EA, Caruso L, Cedroni M, Cotton M, Davidson T, Desai A, Elliott G, Erb T, Fronick C, Gaige T, Haakenson W, Haglund K, Holmes A, Harkins R, Kim K, Kruchowski SS, Strong CM, Grewal N, Goyea E, Hou S, Levy A, Martinka S, Mead K, McLellan MD, Meyer R, Randall-Maher J, Tomlinson C, Dauphin-Kohlberg S, Kozlowicz-Reilly A, Shah N, Swearengen-Shahid S, Snider J, Strong

JT, Thompson J, Yoakum M, Leonard S, Pearman C, Trani L, Radionenko M, Waligorski JE, Wang C, Rock SM, Tin-Wollam AM, Maupin R, Latreille P, Wendl MC, Yang SP, Pohl C, Wallis JW, Spieth J, Bieri TA, Berkowicz N, Nelson JO, Osborne J, Ding L, Meyer R, Sabo A, Shotland Y, Sinha P, Wohldmann PE, Cook LL, Hickenbotham MT, Eldred J, Williams D, Jones TA, She X, Ciccarelli FD, Izaurralde E, Taylor J, Schmutz J, Myers RM, Cox DR, Huang X, McPherson JD, Mardis ER, Clifton SW, Warren WC, Chinwalla AT, Eddy SR, Marra MA, Ovcharenko I, Furey TS, Miller W, Eichler EE, Bork P, Suyama M, Torrents D, Waterston RH and Wilson RK 2005 *Nature* **434(7034):**724-31

115. Porter IM, McClelland SE, Khoudoli GA, Hunter CJ, Andersen JS, McAinsh AD, Blow JJ and Swedlow JR 2007 *J Cell Biol* **179(2):**187-97.

116. Aravind L and Landsman D 1998 *Nucleic Acids Res* **26(19):**4413-21.

117. Onate SA, Prendergast P, Wagner JP, Nissen M, Reeves R, Pettijohn DE and Edwards DP 1994 *Mol Cell Biol* 14:3376-3319

118. Falvo JV, Thanos D and Maniatis T 1995 *Cell* **83:**1101-1111

119. Girard E, Bello B, Laemmli UK and Gehring WJ 1998 *EMBO J* **17:**2079-2085

120. Rasala BA, Orjalo AV, Shen Z, Briggs S and Forbes DJ 2006 *Proc Natl Acad Sci USA* **103(47):**17801–17806

121. Gillespie PJ, Khoudoli GA, Stewart G, Swedlow JR and Blow JJ 2007 *Curr Biol* **17(19):**1657-62

122. Galy V, Askjaer P, Franz C, López-Iglesias C and Mattaj IW 2006 *Curr Biol* **16(17):**1748-56.

123. Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C and Mortensen P 2006 *Cell* **127:** 635-648

124. Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER III, Hurov KE and Luo J 2007 *Science* **316:**1160-1166

125. Kastan MB, Onyekwere O, Sidransky D, Vogelstein B and Craig RW 1991 *Cancer Res* **51:**6304-6311

126. Cortez D, Wang Y, Qin J, Elledge SJ 1999 *Science* **286:** 1162-6.

127. Nakayama M, Nakajima D, Nagase T, Nomura N, Seki N and Ohara O 1998 *Genomics* **51**: 27-34

128. Ruault M, Brun M-E, Ventura M, Roizes G and De Sario A 2002 *Gene* **284**: 73-81

129. Nagase T, Ishikawa K, Miyajima N, Tanaka A, Kotani H and Nomura N 1998 *DNA Res* **5:**31-39

130. Nakajima-Iijima S, Hamada H, Reddy P and Kakunaga T 1985 *Proc Natl Acad Sci USA* **82:**6133-6137

131. Chen H, Wu Y, Zhang Y, Jin L, Luo L, Xue B, Lu C, Zhang X and Yin Z 2006 *FEBS Lett* **580(13)**:3145-52

132. Nagase T, Seki N, Tanaka A, Ishikawa K and Nomura N 1995 *DNA Res* **2**: 167-174

133. Wang ZG, White PS and Ackerman SH 2001 *J Biol Chem* **276:**30773-30778

134. Foderà D, D'Alessandro N, Cusimano A, Poma P, Notarbartolo M, Lampiasi N, Montalto G and Cervello M 2004 *Ann N Y Acad Sci* **1028:**440-9

135. Cheong E, Ivory K, Doleman J, Parker ML, Rhodes M and Johnson IT 2004 *Carcinogenesis* **25(10):**1945-52

136. DeWitt DL and Meade EA 1993 *Arch Biochem Biophys* **306(1):**94-102

137. Kamei D, Murakami M, Nakatani Y, Ishikawa Y, Ishii T and Kudo I. 2003 *J Biol Chem* **278(21)**:19396-405

138. Morita I, Schindler M, Regier MK, Otto JC, Hori T, DeWitt DL and Smith WL 1995 *J Biol Chem* **270(18)**:10902-8

139. Futaki N, Yoshikawa K and Hamasaka Y 1993 *Gen Pharmacol* **24**:105–110

140. Sheng H, Shao J, Kirkland SC, Isakson P, Coffey RJ, Morrow J, Beauchamp RD and DuBois RN 1997 *J Clin Invest* **99(9)**: 2254–2259

141. Subbaramaiah K, Telang N, Ramonetti JT, Araki R, DeVito B, Weksler, BB and Dannenberg AJ 1996 *Cancer Res.* **56**:4424–4429

142. Kargman SL, O'Neill GP, Vickers PJ, Evans JF, Mancini JA and Jothy S 1995 *Cancer Res* **55**:2556–2559