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SUBCELLULAR LOCALIZATION AND MEMBRANE ASSOCIATION OF THE PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE ISOZYMES

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Major professor

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SUBCELLULAR LOCALIZATION AND MEMBRANE ASSOCIATION OF THE PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE ISOZYMES

By

Andrew Gibson Spencer

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ABSTRACT

SUBCELLULAR LOCALIZATION AND MEMBRANE ASSOCIATION OF THE PROSTAGLANDIN ENDPEROXIDE H SYNTHASE ISOZYMES

By

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Using immunoelectron microscopy and photoaffinity labeling we have determined the subcellular locations and membrane association regions, respectively, of prostaglandin endoperoxide H synthase-1 and -2 (PGHS-1 and PGHS-2). PGHS-1 and -2 are the targets of aspirin and are integral membrane proteins of the endoplasmic reticulum and nuclear envelope. Previous experiments in our laboratory led to the hypothesis that these enzymes may reside, at least in part, in different subcellular compartments and that their compartmentation may affect their access to arachidonic acid and serve to separate the functions of the enzymes. High-resolution immunolocalization studies demonstrated that PGHS-1 and -2 were found on the lumenal surfaces of the ER and nuclear envelope in murine NIH 3T3 cells, human monocytes, and human umbilical vein endothelial cells. Within the nuclear envelope, PGHS-1 and -2 were present on both the inner and outer nuclear membranes and in similar proportions. Western blotting data showed a similar distribution of PGHS-1 and -2 in subcellular fractions. Thus, we are unable to attribute the independent functioning of PGHS-1 and -2 to differences in their subcellular locations. A further conclusion of importance from a cell biological perspective is that membrane proteins of the ER such as PGHS-1 and PGHS-2, which are located on the lumenal surface of the ER, are able to diffuse freely among the ER and the inner and outer nuclear membranes of the

nuclear envelope.

PGHS-1 and -2 are integral membrane proteins but do not possess transmembrane domains. Instead, X-ray crystallographic studies led to the hypothesis that these proteins associate with membranes through a novel, monotopic membrane binding domain (Picot, D., et al. (1994) *Nature*, **367**:243-249). Early studies from our laboratory demonstrated that the membrane binding domain of ovine PGHS-1 resides between amino acids 25-166 (Otto, J.C. and Smith, W.L. (1996) *J. Biol. Chem.*, **271**:9906-9910). Using the hydrophobic, photoactivatible reagent 3-trifluoro-3-(m-[^{125}I]iodophenyl)diazirine ([^{125}I]-TID) to label the membrane associated domains of PGHSs, we have extended this work and provided biochemical evidence for the membrane association domains of PGHS-1 and PGHS-2. The PGHS-1 and PGHS-2 membrane binding domains reside within residues 74-140 and 59-125, respectivley.

For my family . . .

Dad for paving the way and everything else.

Mom for giving love and life.

Molly, a true kindred spirit.

Matt, my idol and friend.

I miss my lovely mother, and I
I love my busy father (sic).
I know I owe my brother one thing or another.
I hear my sister singing . . .

-James Taylor, *Terra Nova*

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First of all a big thanks to Bill Smith for teaching me the persistence required to do good science. He was patient with me when I was ready to quit early on and was supportive of my repeated trips to New Jersey to do experiments. Dr. Smith demonstrates by example every day what it takes to be successful in this business. There is probably not another lab in the country that could have provided a better environment for me personally as a graduate student, and I truly believe that. So thanks a lot to Dr. Smith and all current and former Smith/DeWitt lab members for making it great.

Special thanks to Dave DeWitt for being a second mentor to me. Dave is always great to talk to about life in general, experimental problems, computers, food, wine, beer, etc. Thanks also for keeping me going when the experiments in New Jersey weren't cooperating.

Another special thanks goes to John Woods at Merck & Co. In John's lab I first learned how to get things to work. I also became more streetwise to the ways of science and Tri-State trains. My experience in John's lab was very positive and I thank him for everything. Other people at Merck whom I owe big are Jeanne for being like a big sister and Marty Springer for stories, wisdom, and the "best pizza in Brooklyn" at Patsy's.

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Finally a word to the memory of those I lost in 1997-98: My grandfather Paul Johnson, friend and stellar human Scott Bishop, and former Hornet Ty Barber. You are missed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

15d-PGJ_2	15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2
5-LO	5-lipoxygenase
$cPLA_2$	cytosolic phospholipase A_2
EFA	essential fatty acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
FLAP	5-lipoxygenase activating protein
Glu C	endoproteinase Glu C
IL-1β	interleukin-1, β isoform
HETEs	hydroxyeicosatetraenoic acids
HUVEC	human umbilical vein endothelial cells
LPS	bacterial lipopolysaccharide
Lys C	endoproteinase Lys C
MBD	membrane binding domain
NE	nuclear envelope
NPC	nuclear pore complex
NSAIDS	non-steroidal anti-inflammatory drugs
PBMCs	peripheral blood mononuclear cells
PG	prostaglandin
PGHS	prostaglandin endoperoxide H synthase
PPAR	peroxisome proliferator activated receptors
PVDF	polyvinylidene fluoride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
$sPLA_2$	secretory phospholipase A_2
SREBP1	sterol regulatory element binding protein 1
Tx	thromboxane
[^{125}I]-TID	3-trifluoro-3-(m-[^{125}I]iodophenyl)diazirine

CHAPTER I

LITERATURE REVIEW

Introduction

The widespread use of aspirin, ibuprofen, and other non-steroidal anti-inflammatory drugs (NSAIDS) has called for a more thorough understanding of the physiological systems in which they work. NSAIDS exert their anti-inflammatory and analgesic effects through the inhibition of prostaglandin endoperoxide H synthase-1 and -2 (PGHS-1 and PGHS-2), two biochemically similar enzymes that catalyze the committed step in the formation of prostaglandins from arachidonic acid [1]. Prostaglandins, along with leukotrienes, belong to a family of oxygenated, 20-carbon lipid signaling molecules called eicosanoids (Fig. 1). These molecules act as local (autocrine or paracrine) hormones to regulate a broad range of physiological processes including vascular smooth muscle dilation, platelet aggregation, renal water reabsorption, parturition, and tumor formation. The prostaglandin biosynthetic activity of PGHS-1 was first isolated over 20 years ago, and the enzyme has been thoroughly characterized biochemically [2, 3]. In the early 1990's, the discovery of PGHS-2 as a *v-src* and tumor promoter inducible immediate early gene precipitated renewed vigor in prostaglandin research and an attempt to determine the physiological need for two PGHSs [4]. Research into the biology of these two enzymes has increased in our understanding of how aspirin works and how prostaglandins act throughout the body to

Both PGHSs are integral membrane proteins of the endoplasmic reticulum (ER) and nuclear envelope (NE). Their product, PGH_2, which results from the addition of two moles of molecular oxygen to arachidonic acid through the sequential cyclooxygenase and peroxidase activities of PGHS-1 and -2, is the precursor to all prostaglandins (Fig. 2). The biologically active prostaglandins (PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2, and thromboxane) are then synthesized from PGH_2 by specific downstream synthases [2]. After their synthesis, prostaglandins are exported from the cell by one or more plasma membrane transporters where they become available to specific G-protein linked receptors. It is these receptors through which prostaglandins exert their effects by triggering second-messenger responses within cells.

Our laboratory uses chemical, cell biological, and biochemical approaches to gather basic information on the signaling mechanisms of prostaglandins. Structural and biophysical studies of PGHS-1 and -2 have led to important advances in our understanding of aspirin action and prostaglandin biology. An important realization has been that despite their similar biochemical properties, , PGHS-1 and PGHS-2 mediated prostaglandin synthesis can lead to different physiological outcomes (Fig. 3)[1]. One line of evidence for the "two systems" model stems from the vastly different expression patterns of the enzymes: PGHS-1 is expressed constitutively in nearly all mammalian cells and is likely an important housekeeping gene. PGHS-2 is normally absent from cells but can be induced by growth factors, inflammatory cytokines, and bacterial endotoxin. Furthermore, even when both enzymes are present in the same cells simultaneously they seem to be differentially available to their substrate, arachidonic acid [Reddy, 1997 #342]. Recently created knockout mice deficient in PGHS-1 and PGHS-2 have markedly different phenotypes, clearly

demonstrating the different physiological roles of the enzymes [Morham, 1995 #341; Langenbach, 1995 #339].

My dissertation research has focused on the subcellular localization and membrane association of PGHS-1 and PGHS-2. Using high-resolution immunocytochemical techniques I have described the subcellular localization of the two enzymes and provided information important to the determination of the mechanisms by which cells separate their activities. Further, my work on the membrane association of PGHS-1 and -2 has provided biochemical evidence for a novel membrane binding domain that traverses a single leaflet of the lipid bilayer.

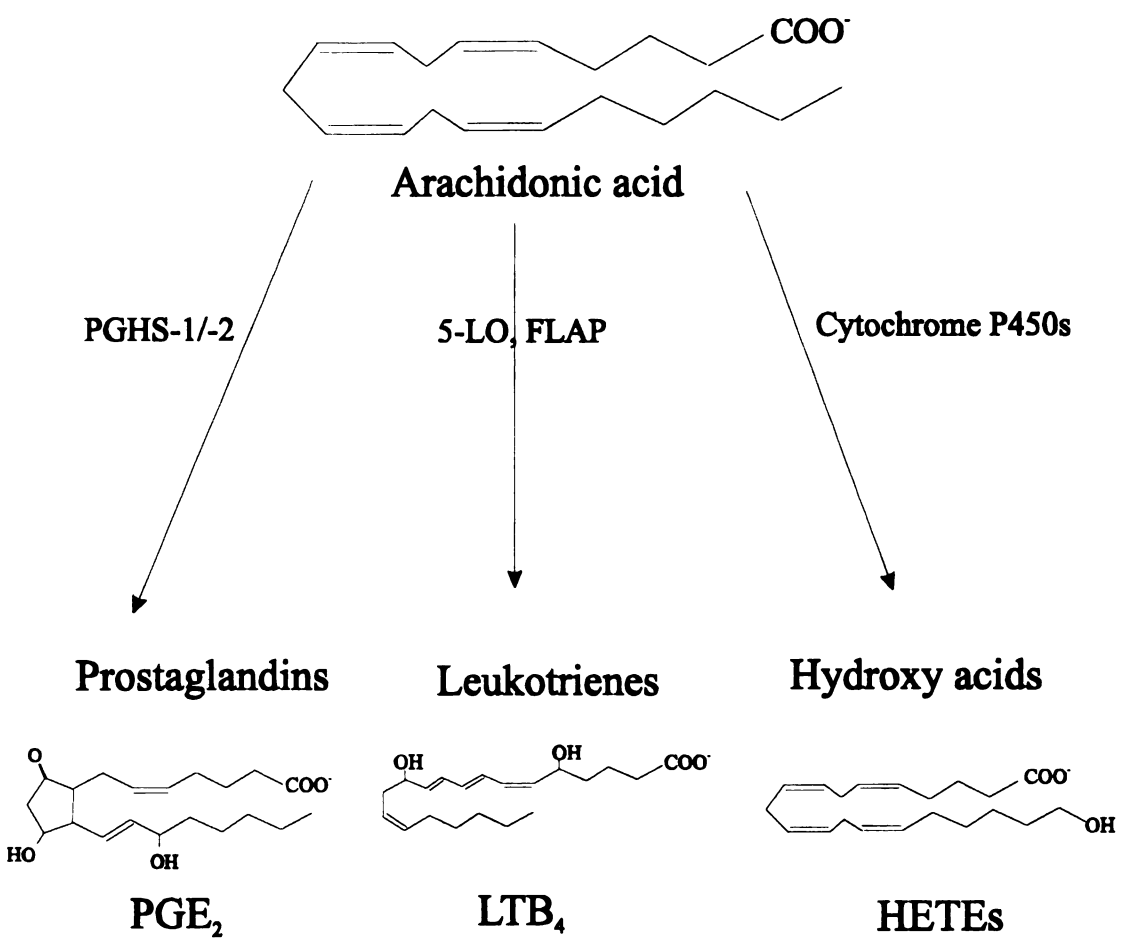


Figure 1. The eicosanoids. Arachidonic acid (20:4) is a component of cellular membranes and is obtained through the diet or via biosynthesis from linoleic acid (18:2). Upon its release from phospholipid stores, this essential fatty acid can be oxygenated and thereby converted to one of several hormones of the eicosanoid family. Members of this family of 20-carbon, lipid-derived hormones include the prostaglandins (e.g., PGE_2), the leukotrienes (e.g., LTB_4), and the hydroxy acids (e.g., HETEs).

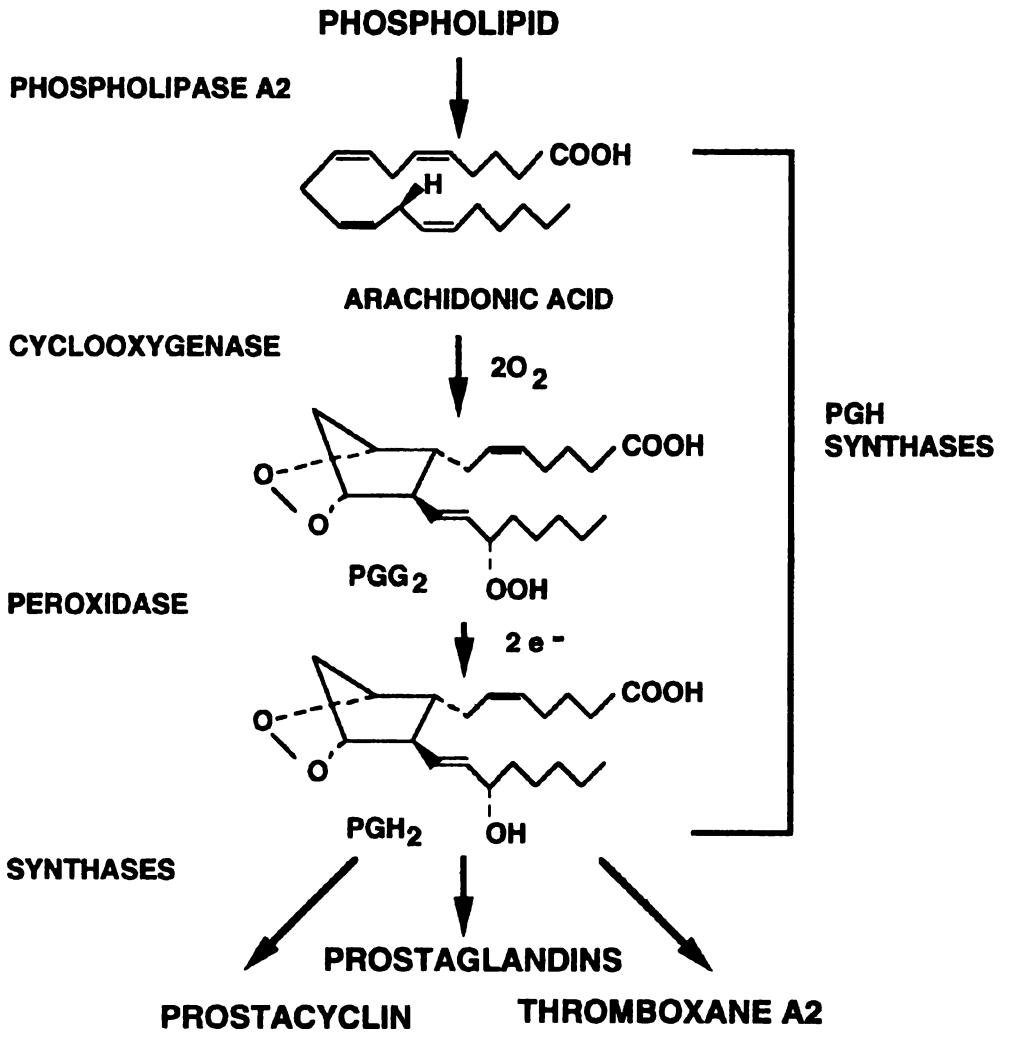


Figure 2. Enzymatic activities of PGHS-1 and PGHS-2. PGHS-1 and PGHS-2 each catalyze the conversion of arachidonic acid to PGH_2 by consecutive cyclooxygenase and peroxidase activities. The cyclooxygenase activity, which is inhibited by aspirin, adds two moles of molecular oxygen per mole of arachidonate to give PGG_2. The peroxidase activity then converts the 15-hydroperoxy group to the corresponding alcohol to yield PGH_2 [1].

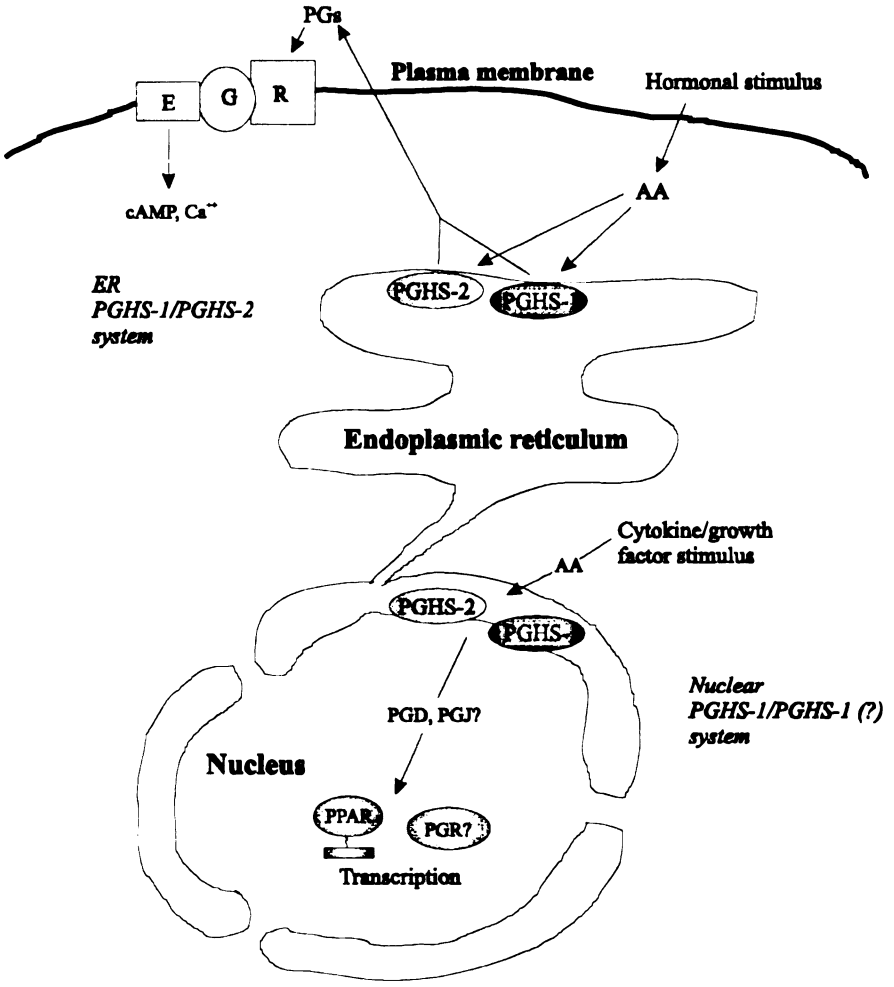


Figure 3. Model of two independent prostaglandin biosynthetic systems. PGHS-1 and -2 are not functionally redundant and may synthesize prostaglandins that subserve different cellular functions. Both PGHS-1 and -2 are known to synthesize prostaglandins that exit the cell and act hormonally via G-protein linked receptors. Due in part to the correlation of PGHS-2 expression with nuclear events like cellular differentiation and mitogenesis, some have hypothesized that a nuclear prostaglandin signaling system exists. Some studies suggest that a prostaglandin may act to modulate transcription by acting as a ligand for the PPAR family of orphan nuclear receptors. *AA = arachidonic acid; PGs = prostaglandins; R- plasma membrane prostaglandin receptor; G = heterotrimeric G protein; E = effector protein; PPAR = peroxisome proliferator activated receptor; PGR? = putative unknown nuclear prostaglandin receptor.*

Historical perspective

Among the landmark studies in vertebrate physiology were those by George and Mildred Burr in 1929 and 1930 demonstrating the dietary essentiality of fatty acids [5, 6]. At the time of their studies, there was general uncertainty regarding the necessity for fats in the diet. During the late 1920's, the Burrs transported a cadre of Long-Evans rats in a Model T Ford from the hills of Berkeley, California to their new laboratory at the University of Minnesota where George was appointed as a plant pathologist in the Department of Botany. When he consulted with the department head – whom he feared might question the need for a plant pathologist to have an animal colony in the Medical school – he was told, "I do not care what field you work in, just do good work in some field." Accordingly, the Burrs began to rigidly exclude fat from the diets of rats over periods of several months, feeding them only protein, carbohydrate, and vitamins A and D. The first obvious effect of dietary fat exclusion was the development of scaly skin, dandruff, and necrosis of the tail. Burr observed that "the early outward signs of an unhealthy condition of the animal are soon followed by a cessation of growth when the animal is about 25% underweight in comparison with the controls receiving fat" [5]. After several months, the rats typically began to lose fur around the face and head and developed sores of the skin. Blood was normally observed in the urine of fat deprived rats. Without exception, rats kept on a fat free diet lost large amounts of weight and died. Autopsies on the dead animals revealed that "the most marked and uniform pathology (was) observed in the urinary tract and the kidney. There seems little doubt that this is an important factor in the death of the animal"[5]. Further study of the fat deprived rats demonstrated a striking lack of fertility when compared to control animals. Interestingly, both the kidney pathology and reproductive problems would be experienced 60 years later

by mice lacking functional PGHS or the appropriate prostaglandin receptors [7, 8, 167].

All symptoms of the fatty acid deficiency disease could be rescued by very small doses of lard, corn oil, linseed oil, or several other "remedies" containing unsaturated fatty acids. Each of these remedies were later found to contain small amounts of arachidonic acid and large amounts of linoleic acid, the metabolic precursor to arachidonate. When the Burrs showed that purified linoleic acid was just as curative of the fat deficiency disease symptoms as the lard or oils, they concluded that "linoleic acid (and possibly other acids) therefore is an essential fatty acid" [6]. Today we fully appreciate the essentiality of plant-derived unsaturated fatty acids in the mammalian diet. These fatty acids and their derivatives are not only important for cellular membrane structure, they serve as precursors to the prostaglandins and other important signaling molecules throughout the body. The kidney failure of Burr's rats was undoubtedly due in part to the lack of substrate for prostaglandin biosynthesis; endogenous PGE_2 production in fatty acid deficient animals is severely reduced [9].

Elsewhere in the early 1930's, while the Burrs contemplated the significance of their work, Ulf von Euler began his postgraduate work in London. He focused on studying what now may seem the inordinately general topic of "naturally occurring, biologically active compounds" [10]. Later, von Euler would be awarded the Nobel Prize for his work on neurotransmitters and the storage and release of norepinephrine. But in the early 1930's, he was busy making extracts from various animal organs and testing their effect on rabbit blood pressure. After many fruitless excursions, von Euler injected small amounts of human semen and saw a dramatic drop in blood pressure [11]. Similar results were observed in extracts from sheep seminal vesicles and using this tissue von Euler continued on this track,

eventually demonstrating that the active component was soluble in lipid solvents. Regarding this observation, von Euler wrote that the finding of a lipid-soluble signaling factor "was at first rather surprising and actually became the turning point of the work, and as much as one can speak of a discovery this finding was clearly the most important one, since it introduced an entirely new kind of pharmacodynamically active substance occurring naturally in the body" [10]. Prior to von Euler's work two American physicians at Columbia University, Kurzrok and Lieb, were trying out artificial insemination in women. In some cases, injection of the semen into the uterus would cause a violent contraction of the uterine muscle which could be reproduced *in vitro* [12]. Although this was the first demonstrated effect of what would later become known as prostaglandins, Kurzrok and Lieb attributed this contractile activity to acetylcholine and left the door open for von Euler to correctly describe the nature of the active compounds.

Despite having passed through the same Swedish laboratory in 1933, Burr and von Euler did not meet and remained oblivious to the strong connections between their lines of investigation in pre-World War II Europe. The subsequent years would forever link these two areas of basic research and describe a hormonal signaling system that conceivably effects every cell in a mammalian organism. As George Burr wrote of von Euler in 1982, "If we had met (in the 1930s) it is likely that we would have discussed our then current work on essential fatty acids and prostaglandins without noting any significant relationship between the two. Now, 50 years later, these compounds are being treated as a unit for the solution of their biological functions"[13].

Due in large part to World War II, progress in the years following the landmark studies of Burr and von Euler was slow. Both pioneers pursued other avenues of research

more vigorously and a group of scientists at the Karolinska Institute in Stockholm began to gather steam in their quest to isolate and describe the first prostaglandin. By the early 1950's the essentiality of dietary unsaturated fatty acids was becoming well understood. Perhaps the greatest advances in the 1950's were chemical and technical in nature. Several of the essential fatty acids including arachidonate, are present in very small quantities, and not until A.T. James and others pioneered the technique of gas-liquid chromatography was it possible for the metabolic pathways traveled by EFAs after ingestion to be mapped [14]. Early radioisotope incorporations were also useful in these studies.

It was more than 20 years after von Euler coined the term "prostaglandin" (a misnomer, the compounds do not originate in the prostate), that Sjovall and Bergstrom at the Karolinska Institute isolated the first prostaglandins in crystalline form [15]. This study predicted the structures of PGE_1 and $PGF_{1\alpha}$ and preceded the many years of work required to determine the structures of the vast number of arachidonic acid-derived hormones called eicosanoids. The 1960's also bore witness to the belated bridge between the early works of Burr and von Euler as Van Dorp and Bergstrom independently showed that these compounds called prostaglandins were synthesized enzymatically from the nutritionally essential arachidonic acid [16, 17].

Research on the biosynthesis of prostaglandins truly took off in the early 1970's. The number of publications related to prostaglandin biology began to skyrocket [13]. The enzyme implicated in its biosynthesis, prostaglandin endoperoxide H synthase, was first purified to homogeneity from bovine and sheep seminal vesicles in 1976 [3, 18, 19]. Five years earlier, John Vane in London had demonstrated that humankind's most common remedy, aspirin, exerted its action through the inhibition of PGHS [20, 21]. This finding, for

which Vane was later awarded a Nobel Prize along with Bergstrom and Samuelsson, eventually led to an adequate explanation of how drugs such as aspirin and ibuprofen work to reduce inflammation, pain, and fever.

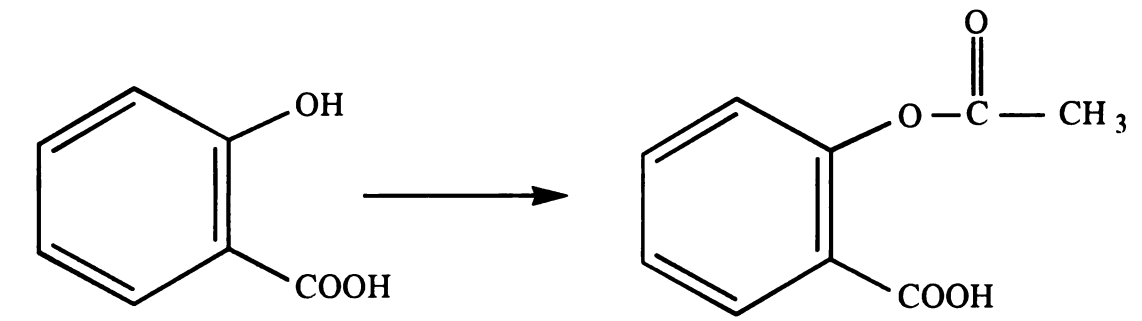
Vane's discovery had a dramatic impact on the medical community. After all, aspirin-like drugs had been in use since the time of Hippocrates 2300 years ago when the great Greek physician had patients chew on white willow leaves to alleviate pain and fever. These willow leaves contained salicylic acid, now a known anti-inflammatory agent. Salicylic acid has a tendency to cause upset stomach in frequent users, and it was this side effect that inspired Felix Hoffman, a German chemist employed by the Bayer Corporation whose arthritic father used salicylic acid, to synthesize aspirin from the original willow compound in 1897. Aspirin is simply an acetylated form of the compound found in the white willow tree. Even today, 100 years after Hoffman's original synthesis, our understanding of aspirin and the physiology associated with its action is continually expanding.

With the 1980s came the molecular biology revolution. Genes for PGHS were cloned [22-24], and site directed mutagenesis studies provided information on which amino acids were important for PGHS catalysis, substrate binding, and aspirin acetylation [2, 25-27]. Much of the enzymatic mechanism was hashed out during this period. Immunocytochemistry was used to localize PGHS-1 to the endoplasmic reticulum and nuclear envelope [28]. In the mid-1980's, Kennedy and others provided the first comprehensive classification scheme for the diverse set of prostaglandin receptors, now known to be members of the G-protein linked receptor family [29, 30]. Each prostaglandin (e.g., PGE_2, PGD_2) binds to one at least one type of specific receptors whose expression

varies according to (a) cell type and (b) the associated second messenger system to which it is coupled.

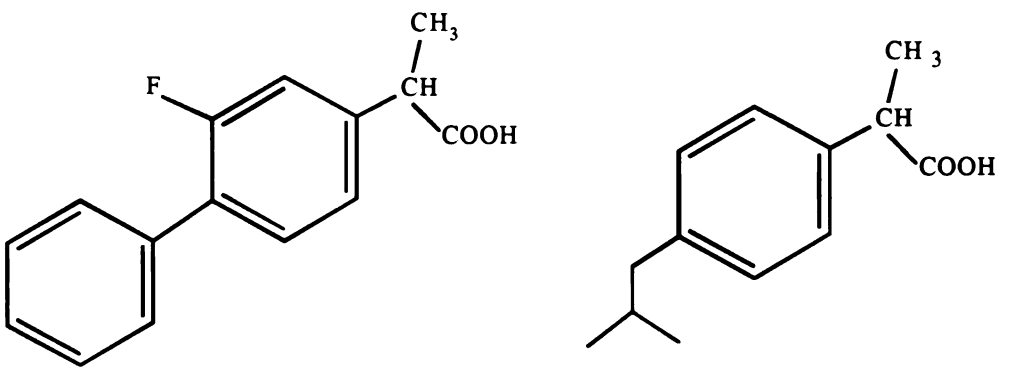
Much of what was known about prostaglandin signaling mechanisms was challenged in 1991 when the second known prostaglandin synthase gene was cloned. PGHS-2 was discovered as an immediate early gene whose expression is induced by phorbol esters and the expression of *v-src* in chicken fibroblasts. Others had previously suggested the existence of two pools of PGHS [31], but proof of the existence of two genes did not come until 1991 in the labs of Harvey Herschman and Dan Simmons [32, 33]. Since then it has become apparent that PGHS-1 and PGHS-2 are not functionally redundant. Instead, they mediate two independent prostaglandin biosynthetic systems in mammalian organisms. Crystallographic analysis of the structures of PGHS-1 and PGHS-2 indicate that these are globular proteins with an associated four helix domain thought to be used for membrane anchoring [34, 35]. Although their crystal structures are virtually superimposable on a global level, important amino acid substitutions around the active site enable the enzymes to use various substrates to different extents and have led to the development of specific inhibitors of each enzyme [26, 36-38].

Perhaps it shouldn't be surprising that mice lacking either PGHS gene exhibited some of the same pathologies as the fat-deprived rats of Burr and Burr 65 years earlier. PGHS-2 knockouts lived only a few weeks before succumbing to severe kidney pathologies and are severely compromised in their reproduction [7]. Other knockout experiments have been performed on genes for prostaglandin receptors. Mice lacking a functional gene encoding the receptor for $PGF_{2\alpha}$ are able to harbor babies in their womb but are unable to deliver the



Salicylic acid
(white willow bark)

Aspirin
(Bayer, Excedrin, Bufferin)



Flurbiprofen

Ibuprofen
(Motrin, Advil)

Figure 4. Structures of some common non-steroidal anti-inflammatory drugs (NSAIDS).

pups (parturition) without induction [167].

The fields of prostaglandin biology and eicosanoids in general are glowing examples of how basic research in the biological sciences can eventually lead to a general understanding of important physiological systems with great clinical relevance. The passage of time lends a perspective unattainable through even the most thorough review of the literature. And as Dr. Burr wrote in 1982, "it also pans the sand and gravel from the gold" [13].

Physical Characteristics of PGHS-1 and PGHS-2

Since PGHS-1 was first purified to homogeneity from seminal vesicles, the genes for PGHS-1 and -2 have been cloned from several species including human, mouse, and rat. Deduced amino acid sequences predict isozymes that share 60% of their primary structure and, when conservative amino acid changes are considered, are 75% similar. The residues required for enzymatic activity are conserved between PGHS-1 and -2 in all species. Both enzymes contain a heme group. PGHS-1 has a molecular weight of 70 kDa, while PGHS-2 exists in both 70 and 72 kDa forms due to differences in glycosylation (see below).

Two clear differences in the primary structures of PGHS-1 and -2 are observed at the N and C termini. Signal peptides in PGHS-1 and -2 differ in their length and share only 25% identity in primary structure. Although these signal peptides are likely to mediate the insertion of PGHS-1 and -2 into the ER during their synthesis, they appear not to confer differential localization nor functional differences of the two enzymes within the ER[1].

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[1]D.L. DeWitt and W.L. Smith, unpublished results.

Another obvious difference between the PGHSs is the 18-amino acid cassette comprising residues 580-598 of PGHS-2. The function of this cassette is unknown. It does not appear to play a role in the subcellular targeting of PGHS-2 but may be involved in targeting PGHS-2 for degradation by cellular proteosomes.

Structures of both PGHS-1 and -2 have been solved by X-ray diffraction of crystals of purified, soluble enzyme [34, 35]. Globally, the structures are essentially superimposable and exhibit a largely α-helical secondary structure. As predicted from early crosslinking and ultracentrifugation studies [18, 39], the crystal structures indicate that both enzymes exist as head-to-tail homodimers. Three major domains are clearly visible in each monomer (Fig. 6). An N-terminal epidermal growth factor (EGF)-like domain is tethered by three disulfide bridges and may play a role in the dimerization of the monomers. The globular catalytic domains of PGHS-1 and -2 encompass both the cyclooxygenase and peroxidase active sites which neighbor the heme site on opposite sides.

The third domain is a novel region predicted to be the membrane binding domain (MBD) of the PGHSs and consists of four amphipathic α helices. These helices form the opening to the cyclooxygenase active site channel where arachidonate is assumed to bind prior to catalysis. From the MBD protrude several hydrophobic amino acid side chains that are likely to interact with the hydrophobic environment of the membrane bilayer [34]. PGHS-1 and 2 are integral membrane proteins, but the MBD is not a classical transmembrane domain. It is predicted that the four helices traverse a single leaflet of the membrane bilayer. Recently, the crystal structure of a bacterial squalene cyclase was solved revealing a PGHS-like putative membrane binding domain [40]. Despite the lack of sequence homology between the squalene cyclase and the PGHSs, it seems probable that this

structural motif may be used by many integral membrane proteins.

Over 8% of the mass of the PGHSs is carbohydrate. Studies by Otto and Smith showed that of four consensus N-glycosylation sites (Asn-XS/T), three (Asn 68, Asn 144, and Asn 410) are glycosylated in ovine PGHS-1 [41]. The fourth, Asn-104, is not glycosylated most likely due to its presence in the putative membrane binding domain where it is sterically unavailable to the glycosylation machinery. The three consensus sites in PGHS-1 are conserved in PGHS-2 and are presumed to be glycosylated in the second isozyme based on electrophoretic mobility. In addition, PGHS-2 has two auxiliary consensus N-glycosylation sites. One of these, Asn-580, is glycosylated in approximately 50% of PGHS-2 molecules and results in PGHS-2 running as a doublet on SDS-PAGE. The function of the PGHS glycosylations are unknown, but mutations in any of the three conserved glycosylation sites in PGHS-1 and PGHS-2 result in less active or inactive enzymes. In contrast, Asn-580 in PGHS-2 can be mutated without loss of activity. All known N-glycosylations take place in the lumen of the ER. Taken together with the mutational analysis of the N-glycosylation sites, this suggests that PGHS-1 and -2 are present on the lumenal surface of the ER.

The possibility of a lumenal orientation was tested directly using immunocytochemistry [42]. Using detergents to selectively permeabilize cellular membranes, immunostaining with multiple antibodies showed that PGHS-1 and -2 are located within the ER lumen. Despite some published evidence to the contrary , the fact that both enzymes are N-glycosylated in cells coupled with the immunofluorescence work provide strong evidence that both PGHSs are present on the lumenal surface of the ER and nuclear envelope.

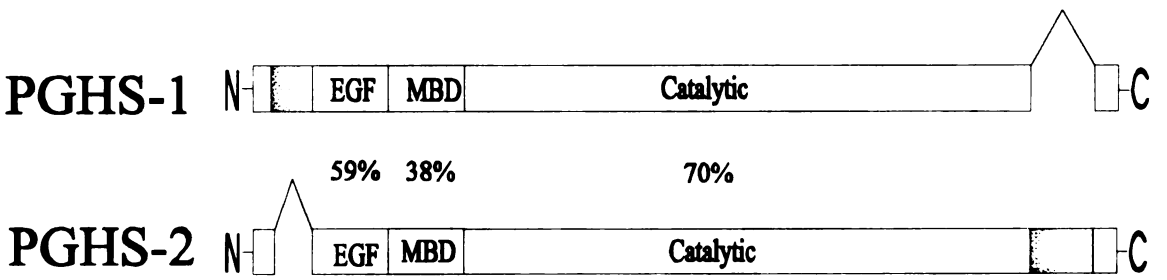

Figure 5. Comparison of the PGHS-1 and PGHS-2 proteins. Percentages denote the sequence identity shared between PGHS-1 and PGHS-2 in the various structural domains. Shaded regions depict short inserts in PGHS-1 and -2 that do not appear in the other isozyme. *EGF = endothelial growth factor domain; MBD = putative membrane binding domain; Catalytic = catalytic domain, which includes the cyclooxygenase and peroxidase active sites along with the aspirin acetylation region.*

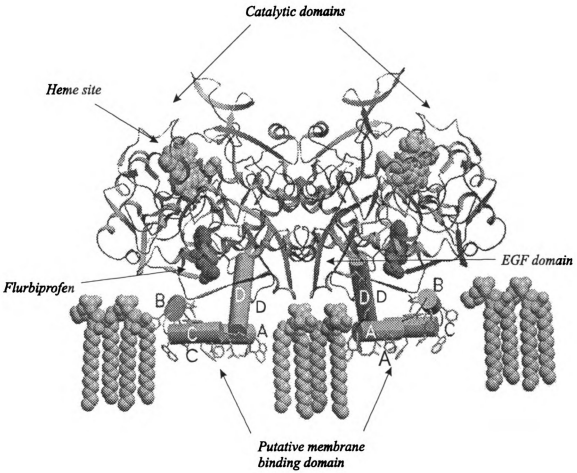

Figure 6. Crystal structure of ovine PGHS-1. PGHS-1 exists as a head to tail homodimer with three major domains: the EGF domain, catalytic domain, and putative membrane binding domain [34]. The structure of PGHS-2 is similar from this view. However, there areseveral non-conservative amino acid substitutions near the active site that result in differential interactions with various non-steroidal anti-inflammatory drugs and fatty acid substrates. Both PGHS-1 and PGHS-2 contain four amphipathic helices (A, B, C, and D above) that are hypothesized to interact with a single leaflet of the lipid bilayer. The four helices of the membrane binding domain form the opening of a hydrophobic channel that leads directly toward the heme site where the enzymatic chemistry takes place. Modified from Ref. 1.

Regulation of PGHS-1 and PGHS-2 expression

The most striking difference in the biology of PGHS-1 and PGHS-2 can be found in their patterns of expression. PGHS-1 is expressed constitutively in nearly all mammalian tissues and forms prostanoids central to several housekeeping functions including water reabsorption in the kidney, vascular homeostasis, and platelet aggregation via the production of thromboxane. PGHS-2 protein, while absent from most cells, can be rapidly and dramatically induced in many cell types upon treatment with inflammatory cytokines, growth factors, and tumor promoters. Thus, PGHS-1 and -2 are often referred to as the constitutive and inducible PGHSs, respectively. However, the "constitutive" and "inducible" labels for PGHS-1 and PGHS-2 do not always apply.

The promoter in the PGHS-1 gene, like many constitutively expressed "housekeeping" genes, does not contain a TATA box, and the 5' region of the gene cannot dramatically activate the transcription of some reporter genes in transfection assays [43]. Culturing NIH 3T3 cells express PGHS-1 at relatively constant levels under many different stimulation conditions. In living mammals, however, the developmental regulation of PGHS-1 is important to normal physiology. For instance, castrated male sheep will not develop functional seminal vesicles expressing PGHS-1, the major membrane protein in this tissue. This effect can be reversed with testosterone treatment, suggesting a role for androgens in the developmental regulation of PGHS-1 in this tissue [44]. In addition, newborn lambs up regulate PGHS-1 at birth to produce a bolus of PGI_2 in their pulmonary artery, presumably to aid in opening the airways to oxygen for the first time [45].

Another tissue in which PGHS-1 is up regulated developmentally is in inflammatory cells like monocytes and macrophages. PGHS-1 is present in monocytes at very low levels.

As they differentiate into macrophages, PGHS-1 is significantly unregulated. A common feature of the developmental up regulation of PGHS-1 in hematopoetic cells is its permanence. The enzyme is unregulated once and presumably becomes a constitutive component of the mature cell or tissue. Basal levels of PGHS-1 expression may be modulated as cells progress through differentiation pathways.

Despite consistent observations that PGHS-1 protein levels do not change dramatically under different culture conditions, it is now clear that its expression is actively regulated *in vivo* in cell and tissue-specific manners. For instance, endothelial cells up regulate PGHS-1 transcription upon treatment with PMA under the likely control of an Sp1 binding site [46, 47].

In contrast to PGHS-1, the up regulation of PGHS-2 is most often rapid and transient in nature. PGHS-2 is classified as an immediate early gene; i.e., its expression does not require the synthesis of new protein. Although generally considered an inducible gene, PGHS-2 is present constitutively in some tissues, most notably the brain and kidney. Immunohistochemical staining of normal rat brain has shown discrete depots of PGHS-2 protein in various regions of the brain, including neurons [48]. Based on the regions of the brain that exhibited PGHS-2 immunostaining, these studies suggest that PGHS-2 may play a role in special sensory input and in the elaboration of behavioral responses. Experiments in knockout mice have shown that the expression of PGHS-2 in the kidney is crucial to mammalian development as PGHS-2 knockout mice die early from acute renal failure [7].

Most tissues do not express PGHS-2 until some stimulus is provided causing a rapid and transient period of PGHS-2 expression. Since PGHS-2 expression is often associated with the inflammatory response, several studies have examined the regulation of this

enzyme in inflammatory cells like neutrophils, monocytes, and mature macrophages. Human monocytes, when treated with low doses of inflammatory cytokines (IL-1β) or bacterial lipopolysaccharide (LPS) begin to up regulate PGHS-2 and synthesize prostaglandins after 12-14 hours [163-165]. This lag period is long compared to other cell types. For example, PGHS-2 expression can be induced within 3 hours in serum-starved NIH 3T3 fibroblasts by the addition of 10% fetal calf serum [49]. The delay in monocytes may indicate the requirement of a secondary autocrine response in addition to the primary IL-1β stimulation.

Up regulation of PGHS-2 expression is likely to occur through several signal transduction pathways. Stimuli that induce the expression of the enzyme vary depending on the cell type being examined. For example, PGHS-2 mRNA accumulates in fibroblasts upon treatment with growth factors and tumor promoters [49]. In macrophages, inflammatory cytokines and LPS trigger its induction. Mast cells require the aggregation of IgE receptors at the cell surface [50]. These and other stimuli are likely to converge on the regulatory regions of the PGHS-2 promoter via several signal transduction pathways including JAK-STAT, p38 map kinase, and protein kinase C pathways [4]. The cyclic AMP response element (CRE) located between nucleotides -56 and -48 upstream of the PGHS-2 transcriptional start site has been shown to play an important role in the transactivation of the PGHS-2 gene. This was shown by transfecting a deletion series of the PGHS-2 promoter/luciferase reporter construct along with a *v-src* expression vector into NIH 3T3 cells [51]. Gel shift analysis of mutations made in this CRE demonstrated that it is indeed the CRE, and not a neighboring E-box, which is responsible for v-src mediated PGHS-2

induction. Similar results have been obtained through the analysis of the human PGHS-2 promoter in monocytic cells [52]. Many other putative response elements exist in the PGHS-2 promoter (e.g. AP-1, NF-kβ, and SRE elements) but have not been shown to be of functional significance.

Many patients suffering from chronic inflammation are prescribed corticosteroids if common NSAIDS like aspirin fail to alleviate their symptoms. At least part of the anti-inflammatory activity of corticosteroids is derived from their ability to inhibit the expression of PGHS-2 protein. Serum- or phorbol ester-induced transcription of the PGHS-2 gene in NIH 3T3 cells is blocked by dexamethasone [49]. Removal of the physiological source of glucocorticoids (the adrenal gland) in rats results in increased levels of PGHS-2 (but not PGHS-1) mRNA and protein in peritoneal macrophages [53]. Treatment of rats lacking adrenal glands with corticosteroids suppressed this accumulation of PGHS-2, demonstrating that adrenal steroids modulate the expression of PGHS-2 *in vivo*.

Aberrant Regulation of PGHS-2 and Implications for Cancer

Failure to properly regulate PGHS-2 expression may play a role in the development of certain cancers. The early evidence for this was circumstantial. For instance, normal colonic epithelia does not express PGHS-2, but a significant number of cancerous lesions in the same tissue do express the enzyme. Epidemiologic data show that patients who consume aspirin regularly are reportedly 50% less likely to die from colon cancer [54]. Recently, clues to the molecular basis for these observations have come from studying apoptosis in colonic epithelial cell lines and from *in vivo* genetic studies using knockout mice. Tsujii, et al. observed that the overexpression of PGHS-2 in colonic epithelial cell

lines impairs the apoptotic response and increases cell adhesion to extracellular matrix proteins. This suggested that the overexpression of PGHS-2 can lead to phenotypic changes that may enhance cells' potential for tumorigenesis [55].

The congenital human disease familial adenomatous polyposis (FAP) is the result of mutations in the APC gene [56]. Patients with this disease develop hundreds of colonic polyps, many of which progress to carcinoma. A mouse model of this disease was recently used to demonstrate the role played by PGHS-2 in neoplastic transformation. Like FAP patients, mice lacking a functional APC gene spontaneously accumulate hundreds of precancerous polyps in their colonic epithelium. In the recent study, APC-deficient mice were crossbred with mice lacking functional PGHS-2. The striking result was a loss of 86% of the precancerous lesions in the APC/PGHS-2 double knockout compared to the APC knockout [57]. When the APC-deficient mice were treated with specific inhibitors of PGHS-2 a similar reduction in precancerous polyps was observed providing genetic and pharmacological evidence that PGHS-2 activity is involved in the neoplastic transformation of colonic epithelium.

Similar results have been observed in humans. FAP patients experience a drastic reduction in the size and number of cancerous polyps when treated with the NSAID sulindac [58]. It seems likely that a significant number of available NSAIDS may be broadly effective in preventing colon cancer, although controlled human studies are lacking. In any event, aberrant regulation of PGHS-2 has clearly been implicated in the early steps of APC-mediated neoplastic transformation.

Mobilization of Arachidonic Acid and its Availability to PGHS-1 and PGHS-2

An important step in the biosynthesis of prostaglandins is the release of arachidonic acid from phospholipid stores. Arachidonic acid is preferentially found at the *sn*-2 position of glycerophospholipids and is cleaved by one of a number of phospholipases A_2 (PLA_2s) to generate "free" arachidonate and lysophospholipids. Whether or not "free" arachidonate exists within a cell is questionable. Several fatty acid binding proteins (FABPs) have been cloned, and thus it is conceivable that there are proteins that shuttle arachidonate within the cell after its release from phospholipid stores. The number of identified PLA_2s is growing steadily (now over ten), and all are classified according to molecular weight, amino acid sequence, and structural homology [59, 60]. In addition to providing arachidonate to the prostaglandin biosynthetic machinery, PLA_2's also provide this substrate to the other major arm of the arachidonic acid cascade, the leukotriene biosynthetic system. Despite their common function of releasing arachidonic acid from membranes, the physiological roles of different PLA_2's appear distinct and different PLA_2s may be independently coupled to PGHS-1 or PGHS-2.

Cytosolic PLA_2 ($cPLA_2$) is an 85-kDa enzyme that when activated preferentially hydrolyzes arachidonate from the *sn*-2 position of phosphoglycerides. In resting cells $cPLA_2$ is soluble and inactive. Agonist-induced increases in intracellular calcium levels (from 50 nM to 0.3-1.0 μM) activate $cPLA_2$ by promoting its association with membranes, presumably the ER and nuclear envelope [61, 62]. A further level of $cPLA_2$ regulation is achieved through phosphorylation. Ser-505 phosphorylation *in vivo*, which may be mediated by members of the MAP kinase family [63-65], results in an increase in $cPLA_2$ activity.

Expression of $cPLA_2$ is nearly ubiquitous, but it is enriched in cells involved in the inflammatory and allergic responses.

$cPLA_2$ is active at the nuclear envelope and was recently shown to be expressed in the nucleoplasm of endothelial cells under certain conditions [59, 66]. Several eicosanoid biosynthetic enzymes, including PGHS-1, PGHS-2, 5-lipoxygenase, and 5-lipoxygenase activating protein have a demonstrated association with the nucleus [67-70]. The subcellular locations of these proteins strongly suggest that the functional components required for eicosanoid biosynthesis can assemble on the nuclear envelope.

The other major group of PLA_2s are the 14 kDa secretory PLA_2s ($sPLA_2$s). These enzymes also require calcium but use it for the catalytic mechanism of arachidonate release rather than for membrane association. Five mammalian 14 kDa PLA_2s have been characterized (Group IB, group IIA, group IIC, group V, and the calcium-independent group VI) and are distinguished by their number of disulfide bridges, expression patterns, and physiological roles [60]. Although they are not specific for arachidonic acid at the *sn*-2 position like the 85-kDa $cPLA_2$ (e.g., $sPLA_2$s will hydrolyze arachidonic and linoleic acid from the *sn*-2 position with equal efficiency), the 14 kDa PLA_2s hydrolyze significant amounts of arachidonate from inflammatory cells, lung, and synovial fluid. Depending on the cell type, the $sPLA_2$'s seem to play important roles in the release of arachidonic acid by activated inflammatory cells (see below).

The 14kDa PLA_2s must interact with the plasma membrane for activity *in vivo*. Recent work by Mike Gelb's group at the University of Washington used biophysical techniques to show that a patch of hydrophobic residues conserved in all $sPLA_2$'s forms a binding surface through which the enzymes interact with membranes [71]. Interestingly, the

$sPLA_2$'s apparently require a perturbation of cellular membranes for activity, as their activity on intact membranes is low [72, 73]. Consistent with these observations, the ability of $sPLA_2$s to release arachidonate from the plasma membrane is highly dependent on the activation state of the cells being studied. Exogenous addition of $sPLA_2$ to macrophages does not result in prostaglandin synthesis unless the cells have been pretreated with lipopolysaccharide and platelet activating factor [74].

The simple presence of PGHS-1/-2 and a phospholipase A_2 in the cell is not sufficient in many cases to achieve prostaglandin synthesis. In fact, PGHS-1 and -2 are often differentially available to arachidonic acid when expressed simultaneously in the same cells. For example, arachidonate can be released into RAW 264.7 cells expressing both PGHSs and only PGHS-1 can use it to make prostaglandins [75]. In addition, cell lines from which the gene for PGHS-1 has been deleted express high levels of PGHS-2, but cannot synthesize prostaglandins from arachidonic acid added exogenously. Conversely, PGHS-2 knockout cell lines express PGHS-1 and can make prostaglandins from arachidonic acid added to the cells [76]. The observation that not all arachidonate is indiscriminately available to the PGHSs led to the idea that there were two cellular "pools" of arachidonate, one for PGHS-1 and one for PGHS-2. A more likely explanation for these observations is that distinct PLA_2's are functionally coupled to either PGHS-1 or PGHS-2.

The possibility of PLA_2 coupling to PGHS-1 or -2 is being studied in several laboratories using activated inflammatory cells, which can release arachidonate and synthesize prostaglandins in distinct early and late phases. The early phase releases arachidonate within the cell and occurs during the first 10-20 minutes of stimulation resulting in prostaglandin synthesis via PGHS-1 . The late phase, extracellular release of

arachidonate persists for several hours and is mediated by PGHS-2 [50, 77]. Attempts to determine the PLA_2's responsible for the early and late phases of arachidonate release have revealed a complex and often contradictory picture. For example, Reddy, et al. found that early and late phase arachidonate release was mediated by type IIA $sPLA_2$ and $cPLA_2$, respectively [78]. Earlier, Bingham et al. had observed the converse [79]. Conflicting results such as these may be explained by differences in both the condition of the cells and the stimulation used. Other labs have since performed similar experiments, and is most cases it seems that $cPLA_2$ releases arachidonate intracellularly for early phase prostaglandin synthesis while $sPLA_2$ mediates the extracellular, late phase burst of arachidonate release and prostaglandin synthesis. This model is consistent with the cell biological properties of $cPLA_2$ and the $sPLA_2$'s, which bind to intracellular and extracellular membranes, respectively, when activated.

The early studies mentioned above indicated that the type IIA $sPLA_2$ played a central role in PGD_2 synthesis by stimulated mast cells [77, 79]. However, recently discovered null mutations in the type IIA $sPLA_2$ gene were shown to have no effect on the patterns of arachidonic acid release and PGD_2 synthesis. Instead, the newly cloned type V $sPLA_2$ appeared to be the relevant enzyme in late phase mast cell arachidonate release [80]. A similar reassessment of the roles of the type IIA and type V $sPLA_2$s in mouse macrophages was performed in Ed Dennis' lab at the University of California at San Francisco [81, 82]. The misidentification of type IIA $sPLA_2$ in inflammatory cell arachidonate release was likely due to activity assays and antibodies that were unable to distinguish between the structurally similar type IIA and type V enzymes. Nevertheless, type IIA $sPLA_2$ is obviously important in some cell types. For instance, rat fibroblasts that do not

express the type V $sPLA_2$ display the typical biphasic prostaglandin production mediated by $cPLA_2$ and type IIA $sPLA_2$ [83]. It may be that under certain conditions the type IIA and type V PLA_2s are functionally redundant [84].

There may be crosstalk between $cPLA_2$ and type V $sPLA_2$ that is essential to the coordination of early and late phase prostaglandin synthesis in inflammatory cells. As mentioned above, a membrane perturbation event is required before $sPLA_2$ is able to release arachidonate from cellular membranes [72, 73]. One model of this crosstalk system implicates $cPLA_2$-derived arachidonate in the generation of the perturbation event.

Most of the late phase PGE_2 released from stimulated mouse macrophages is derived from PGHS-2. Interestingly, one can prevent this late phase, $sPLA_2$-mediated PGE_2 release by specific inhibition of $cPLA_2$ activity [74]. This effect *cannot* be reversed by exogenously added $sPLA_2$. However, it can be reversed by artificially increasing the intracellular arachidonate levels in the cell prior to activation. These studies, along with recent observations by Kuwata, et al,. suggest that early phase $cPLA_2$-derived arachidonic acid generates a signal that is required for the late phase, $sPLA_2$-mediated release of prostaglandins (Fig. 7) [74, 83].

One rather speculative possibility to account for the independent operation of PGHS-1 and PGHS-2 in cells where both isozymes are expressed is the existence of accessory proteins which differentially affect the rate of prostaglandin endoperoxide formation by PGHS-1 versus PGHS-2. Although no such protein(s) has been identified in the prostanoid biosynthetic system, there is a precedent for an accessory protein in the leukotriene pathway. Leukotrienes synthesized through 5-lipoxygenase arise from arachidonic acid apparently delivered to the 5-lipoxygenase by an activating protein,

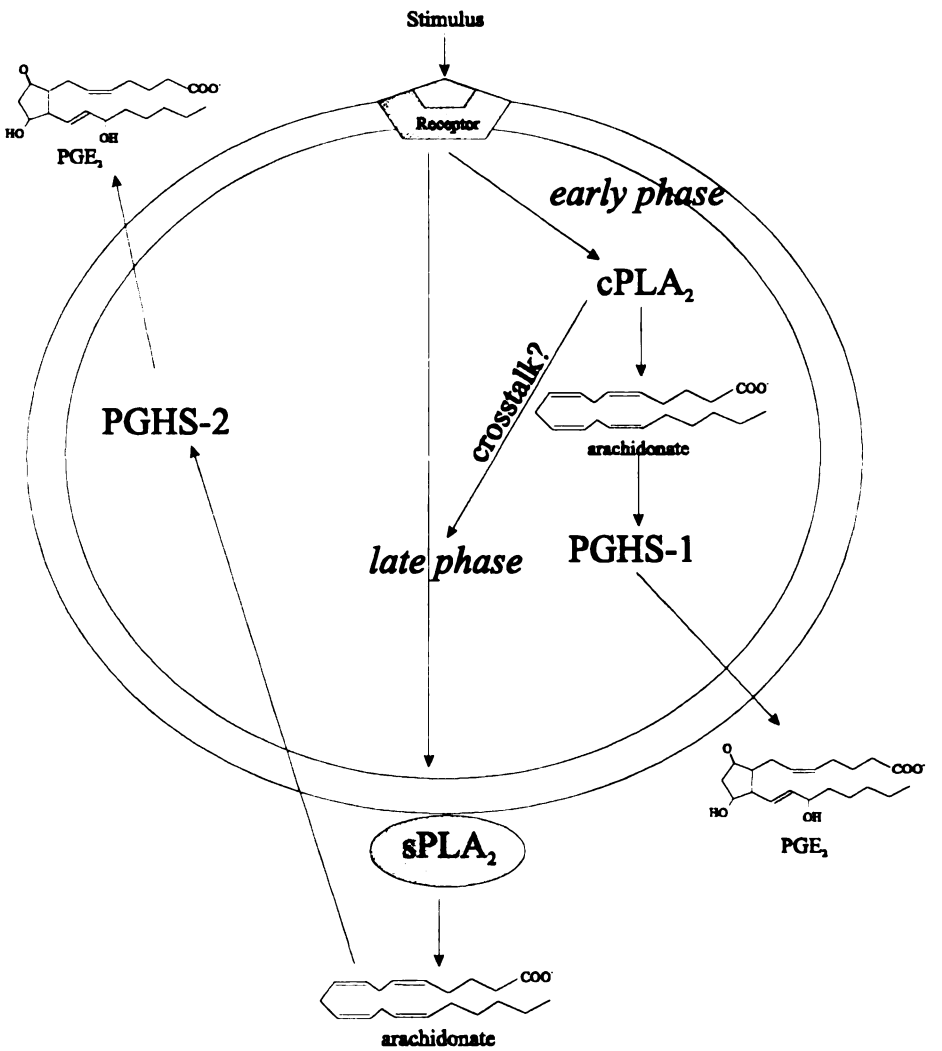

Figure 7. Schematic diagram of the biphasic prostaglandin synthesis in activated inflammatory cells.

FLAP [85, 86].

Prostaglandin Signaling via G-protein Coupled Receptors

Prostaglandins act as short-lived local hormones to maintain homeostasis in the cells from which they were released and their neighbors. Immediately after their synthesis, prostaglandins are released from the cell via at least one known prostaglandin transporter [87, 88]. The family of G-protein coupled receptors through which prostaglandins exert their signaling messages was classified by Kennedy and Coleman in 1983 [29]. Specific receptors exist for PGE_2, PGI_2, PGD_2, $PGF_{2\alpha}$, and thromboxane. The corresponding receptors are known as EP, IP, DP, FP, and TP receptors, respectively, and some subclasses exist (EP1, EP2, etc.) These receptors are coupled to heterotrimeric G-proteins which in turn modulate the activities of effector proteins to control cellular levels of cAMP, Ca^{2+}, and phosphatidyl inositol [89]. Nearly all mammalian tissues possess at least some prostaglandin receptors.

Structurally, all the known prostanoid receptors are thought to resemble the rhodopsin family of seven transmembrane domain receptors based on similarities to known rhodpsin-like structures and hydrophobic sequence analysis [89]. The N-terminal extracellular regions contain several N-glycosylation sites and about 35% of the receptor mass is carbohydrate [90]. Two cysteines in the first and second intracellular loops may provide structural stability through a disulfide bridge. The seventh transmembrane domain of all prostanoid receptors contains a conserved arginine. This residue probably participates in binding a part of the prostaglandins present in all species. Funk, et al. demonstrated in 1993 that a point mutation at this arginine residue eliminates the ability of the thromboxane receptor (TP) to bind its ligand [166]. Intracellularly, a series of Ser and Tyr

phosphorylation sites are thought to serve as desensitization switches like those found in the adrenergic and muscarinic receptors of the rhodopsin family, and some work has been done to demonstrate this [91-95]. Prostaglandin receptors, like PGHS-1 and -2, are important clinical targets and prostaglandins and their analogues are frequently used clinically to combat cardiovascular disease, induce labor in pregnant women, and relieve erectile dysfuntion in impotent men [96].

Prostaglandins are regulatory hormones that often work in opposition to one another to maintain homeostasis in a given tissue. For example, thrombin stimulates platelets to release thromboxane A_2 which causes platelets to aggregate. Balancing this action, thrombin-induced PGI_2 release from endothelial cells inhibits platelet aggregation, thus preventing unnecessary clotting. A further example of the opposing actions of prostaglandins can be observed in vascular smooth muscle. PGE_2 can cause the contraction *or* relaxation of vascular and nonvascular muscles through EP1 and EP2 receptors, respectively, because the two receptors are coupled to different G proteins that mediate opposing actions. Thus, the transcriptional regulation of prostaglandin receptors can significantly affect a tissue's response to a given stimulus.

All the prostanoid receptors bind their respective ligands with binding constants of 1.3-40 nM [89]. At concentrations higher than this, cross reactivity is observed. For instance, PGI can activate the EP_1 receptor as potently as PGE_2 [97]. It is therefore important to take into account the possibility for receptor cross reactivity when studying cells that express more than one PG receptor.

Evidence for Nuclear Prostaglandin Signaling Systems.

The physiological effects of prostaglandins are best understood through their role at the plasma membrane where they bind to specific G-protein coupled receptors. Recently, the idea that prostaglandins act exclusively through extracellular mechanisms has become the subject of debate. Due in part to the correlation of PGHS-2 expression with nuclear events like increased gene transcription and mitogenesis [4], it has been hypothesized that a subset of prostaglandins enter the nucleus and modulate transcription through a group of orphan nuclear receptors. This section will review the evidence for and against the possibility of nuclear prostaglandin signaling mediated by the orphan nuclear receptor, PPARγ.

Peroxisome proliferator activated receptors (PPARα, PPARγ, and PPARδ) are members of the nuclear hormone receptor superfamily [98]. Upon ligand binding, these receptors form a heterodimer with the retinoid X receptor (in the case of PPARγ) which in turn activates the transcription of genes involved in lipid metabolism and biosynthesis. Like other members of the nuclear receptor superfamily, PPARs are comprised of a ligand binding domain, a hinge domain, and a DNA-binding domain. In the case of PPARγ the DNA binding domain binds to a specific 6-nucleotide sequence dubbed DR-1 [99]. The endogenous ligands for the PPARs are unknown, but several synthetic anti-diabetic drugs of the thiazolidinedione family (e.g., troglitazone and BRL49653) can act as ligands *in vivo* and *in vitro* [100]. Despite appreciable sequence homology, the PPAR's exhibit very different tissue distribution and are known to bind ligands with differing affinities.

The biological role of PPARγ is best understood in the context of the differentiation of adipose tissue. This process is controlled by the interplay between PPARγ and other

transcription factors [101]. Since its discovery, the endogenous ligand for PPARγ has been unknown. Recently, some have speculated that a prostaglandin or other eicosanoid may be the endogenous ligand for the orphan nuclear receptor, PPARγ. This hypothesis is based on the ability of certain prostaglandins and unsaturated fatty acids to activate transcription via PPARγ under experimental conditions [102, 103]. Activation of PPARγ with ligands is sufficient to induce adipogenesis in several fibroblast lines [101]. The likelihood that adipose tissue itself has prostaglandin biosynthetic capacity is suspect, but circumstantial evidence is beginning to gather in support of a role for nuclear prostaglandin signaling in other cell types. For instance, stimuli that induce the expression of PGHS-2 in monocytes can also trigger the expression of PPARγ.

Many of the recent studies positing a role for prostaglandins in PPARγ-mediated nuclear signaling have been conducted using cells transfected to express PPARγ. Treatment of PPARγ-transfected NIH 3T3 mouse fibroblasts with the prostaglandin 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 causes their dramatic differentiation to adipocytes [104]. Similarly, genes known to be regulated by PPARγ can be co-transfected into cells along with PPARγ and turned on with exogenous treatment with 15d-PGJ_2. 15d-PGJ_2, is a naturally-occurring metabolite of PGD_2, the major prostanoid product in monocytes and macrophages.

Prostaglandins can also modulate transcription in cells that express PPARγ endogenously. 15d-PGJ_2 can prevent the usual transcription of at least three inflammatory genes in activated mouse peritoneal macrophages [105]. Further, the normal production of inflammatory cytokines by activated monocytes can be inhibited by treatment with 15d-PGJ_2 [106]. In all of the studies mentioned above, the involvement of PPARγ in the observed

phenomena was confirmed by treatment with the known PPARγ ligands troglitazone and BRL, members of a family of anti-diabetic drugs that were some of the first identified PPARγ ligands. However, the case for nuclear action by 15d-PGJ_2 is far from closed.

Prostaglandins bind to their respective plasma membrane receptors with K_d values between 1.3 and 40 nM [89]. Known endogenous ligands for members of the nuclear hormone receptor superfamily like the retinoid X receptor (PPARγ's heterodimerization partner) have K_d's that are typically <1 nM. In contrast, all of the studies purporting 15d-PGJ_2 to be the ligand for PPARγ have used ligand concentrations of at least 100 nM and often higher. At these concentrations of ligand one must take into account at least three considerations: (a) 15d-PGJ_2 may non-specifically activate one of the known plasma membrane receptors (e.g., EP1, EP2, or FP1); (b) there may be cell surface receptors for 15d-PGJ_2 itself; and (c) exogenously administered 15d-PGJ_2 would have to cross the plasma membrane to reach PPARγ. The latter point refers to the fact that prostaglandins are generally not able to diffuse across cellular membranes. Exogenously administered prostaglandins may require a transporter to reach the nucleus.

Bruce Speigelman at Harvard Medical School is currently using a slightly different approach in his search for the endogenous ligand for PPARγ. Instead of treating cells with suspected natural ligands of PPARγ, his group is collecting lipid-soluble molecules in tissue culture supernatants and testing these extracts for the ability to modulate PPARγ -mediated transcription. PPARγ is known to interact functionally with SREBP-1, a protein implicated in fatty acid and cholesterol metabolism [101]. Spiegelman's group recently showed that the expression of SREBP1 specifically increases the transcriptional activity of PPARγ

through its ligand binding domain [107]. This stimulation of PPARγ by SREBP1 does not require co-expression in the same cells; lipids extracted from the supernatants of cells expressing SREBP1 can stimulate the transcriptional activity of PPARγ in separate cultures. Biochemical experiments showed that cells expressing SREBP1 secrete lipid molecules that bind directly to PPARγ.

From these studies, Speigleman's group concluded that SREBP1 stimulates the production of an unknown lipid mediator which can then enter the nucleus and modulate transcription via PPARγ. Here again one should consider that prostaglandins are generally impermeable to cellular membranes. However, some studies of prostaglandin metabolism in the lung have suggested that prostaglandins can indeed be transported into the cell in a specific manner [108]. A similar transport system could conceivably act to facilitate prostanoid access to the nucleus. Although the existence of eicosanoid signaling in the nucleus has not been rigorously described, the localization of the required biosynthetic enzymes and prostaglandin activation of PPARγ provide important leads. Spiegelman's work is interesting and his group is currently attempting to determine the nature of the SREBP-1 regulated factor that activates PPARγ. Preliminary results suggest that it is not 15d-PGJ_2 [107].

The Nuclear Envelope: Structure and Membrane Protein Trafficking

As integral membrane proteins of the ER and nuclear envelope, PGHS-1 and PGHS-2 are interesting in terms of their cell biology and the strategies they use to reach the cellular membranes where their activities are necessary. Immunolabeling studies of PGHS-1 and -2

discussed in Chapter II of this dissertation provide the first evidence that an integral membrane of the ER can reach the inner membrane of the nuclear envelope, presumably by lateral diffusion.

The membranes of the nuclear envelope serve to compartmentalize the nucleus of eukaryotic cells. The inner nuclear membrane, outer nuclear membrane, nuclear pore membranes, and the ER form a continuous membrane system (Fig. 8) [109]. The outer nuclear membrane is essentially part of the ER; any membrane proteins present in the ER are therefore expected to be in the outer nuclear membrane by extension. The inner nuclear membrane contacts the outer nuclear membrane only at the nuclear pore complexes (NPC). Thus, any protein able to reach the inner nuclear membrane must first circumvent the large proteinaceous NPC. The inner nuclear membrane is functionally and biochemically distinct from the outer membrane. Several proteins have now been localized exclusively to the inner nuclear membrane, including the lamin B receptor (LBR), LAP1C, LAP2, and emerin [110-114]. All these proteins play a role in the structural integrity of the nuclear envelope or interphase chromosomes through interactions with chromatin and the nuclear matrix.

A model has emerged to describe how integral membrane proteins become localized to the inner nuclear membrane after their synthesis in the ER [115]. Proteins are thought diffuse laterally in the membrane bilayer from the outer nuclear membrane through the nuclear pore membranes until they reach the inner nuclear membrane. Ellenberg, et al. used fluorescence recovery techniques to provide evidence for the lateral diffusion model using LBR/GFP fusion proteins [116]. This study demonstrated that recently synthesized LBR in the ER could freely diffuse in the lipid bilayer until it reached the inner nuclear membrane

where it became anchored to its ligand. Due to the physical barrier of the NPC, certain types of membrane proteins are prevented from reaching the inner nuclear membrane via lateral diffusion. For example, membrane proteins with large (>70 kDa) cytoplasmic/nucleoplasmic domains fail to reach the inner nuclear membrane (see the Chapter II Discussion) [114]. This observation is likely due to a size constraint imposed by the lateral channel diameter of the NPC.

PGHS-1 and -2 are interesting in this regard. Both proteins have large, globular extramembrane domains, but due to their lumenal orientation in the membrane, can apparently diffuse laterally past the NPC and to the inner nuclear membrane where they may serve to synthesize prostaglandins that enter the nucleus. The PGHSs are (a) the first ER membrane proteins with a demonstrated presence on the inner nuclear membrane, and (b) the first known proteins of the inner nuclear membrane with no known role in the structural integrity of the nucleus or nuclear envelope. Further studies in this area will provide fundamental evidence pursuant to the properties of membrane proteins and their trafficking patterns within cells.

PGHS Catalysis and Inhibition by NSAIDS

PGHSs contain two enzymatic activities. The cyclooxygenase activity catalyzes the bis-oxygenation of arachidonic acid to give PGG_2. The peroxidase activity then reduces the 15-hydroperoxyl group of PGG_2 to form PGH_2 (Fig. 2). The existence of two activities in a single enzyme make the kinetic properties of PGHS-1 and -2 interesting and complex [2]. Crystallographic analysis reveals that although these active sites neighbor each other, they are on opposite sides of the heme and appear to be distinct [34].

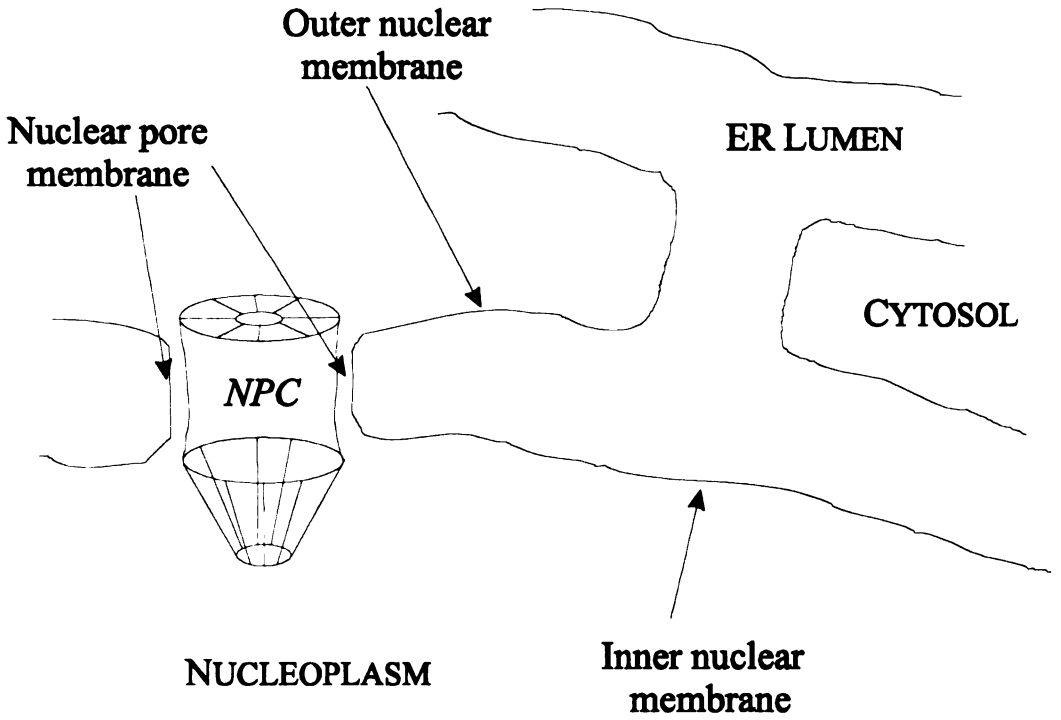

Figure 8. The continuous membrane systems of the ER and nuclear envelope. The endoplasmic reticulum is attached to the nuclear envelope by virtue of its continuity with the outer nuclear membrane. The inner and outer nuclear membranes are separated by the nuclear pore membranes which surround the nuclear pore complexes (NPC). The NPC serves as a barrier to free diffusion of membrane proteins from the outer nuclear membrane to the inner nuclear membrane, and thus keep the inner and outer membranes functionally and biochemically separate. Modified from Ellenberg, et al. 1997.

Following the binding of substrate, cyclooxygenase activity is triggered by a hydroperoxide-driven oxidation of the heme iron at the peroxidase active site (Fig. 9). With its iron atom now in a Fe^{4+} oxidation state, the heme can oxidize Tyr385[2] to create a tyrosyl radical. This radical is thought to perform the rate-limiting abstraction of the 13-*pro S* hydrogen of arachidonic acid. Once this occurs, molecular oxygen is able to attack the substrate at position 11 and form a bridged endoperoxide with carbon 9. A second molecule of oxygen is then added at carbon 15 to give PGG_2, which is quickly reduced at the peroxidase active site to form PGH2.

How the PGG_2 moves from the cyclooxygenase channel to the peroxidase active site is poorly understood. Structural biologists generally agree that it is unlikely PGG_2 moves from the proximal to the distal side of the heme through the protein. Instead, the newly oxygenated substrate probably exits the hydrophobic channel of the cyclooxygenase active site through an unknown route and diffuses to the peroxidase site. The lower region of the active site channel contains one possible exit route between Helix A and the EGF domain (see Fig. 6). Conformational changes in the membrane associated region of the protein may help to facilitate PGG_2 diffusion, and the PGHSs are generally flexible in this region suggesting other exit routes may exist.

Several other amino acids in both PGHS isozymes are required for activity. At the mouth of the cyclooxygenase channel, arginine 120 interacts with the carboxyl group of arachidonic acid to position the substrate for hydrogen abstraction by Tyr385. Similar

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[2]Amino acid numbers correspond to the sequence of ovine PGHS-1.

interactions are important in PGHS-2, but to a lesser extent[3]. The heme group is coordinated by two histidine residues, with His388 and His207 acting as the proximal and distal ligands, respectively [34, 117]. Mutations at these positions result in a loss of both enzymatic activities.

Both PGHS-1 and -2 undergo a poorly understood phenomenon called suicide inactivation. Oxygen uptake assays of preparations of PGHS-1 or -2 show an initial burst of O_2 consumption as PGG_2 is formed that tapers off within two minutes. On average, each enzyme molecule can turnover about 400 times before becoming suicide inactivated. How this inactivation occurs is unknown, but it probably results from the decay of an unstable enzyme-substrate intermediate. Interestingly, some mutations (His386Ala and Arg120Glu) result in an enzyme that does not undergo suicide inactivation [118]. The His 386 residue may be involved in the transport of the radical from neighboring Y385 that may somehow trigger an aberrant cross linking reaction that kills the enzyme.

All common NSAIDs (e.g., aspirin, ibuprofen) inhibit prostaglandin synthesis by binding to or covalently modifying the cyclooxygenase active site of the PGHSs. Aspirin exerts its pharmacological action by acetylating serine 530 in PGHS-1 [25, 119]. PGHS-2 is also acetylated by aspirin but unlike PGHS-1 is not completely prevented from oxygenating the arachidonate and can make 15-HETE [120]. This suggests that the PGHS-2 active site is larger

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[3]C.J. Rieke and W.L.Smith, unpublished results.

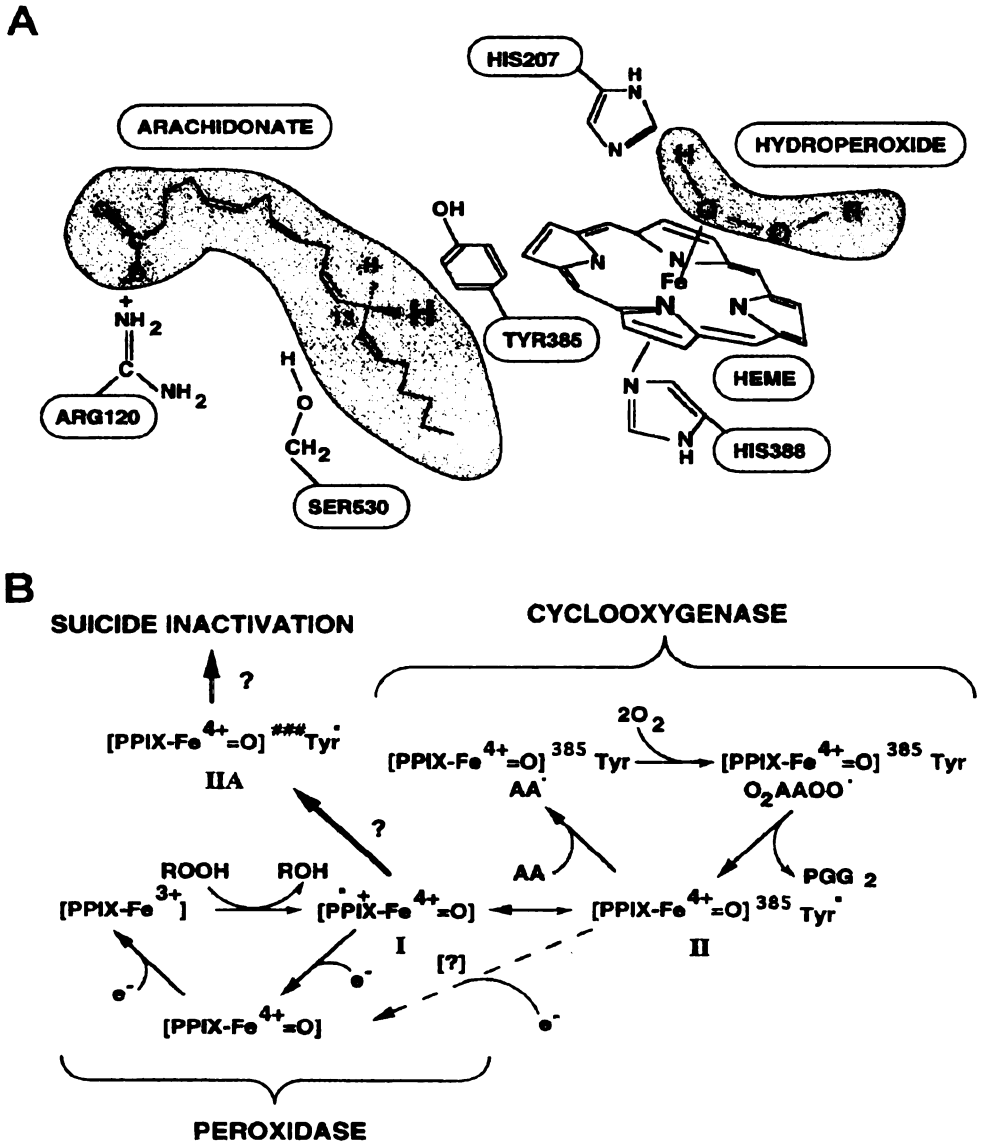


Figure 9. Peroxidase and cyclooxygenase catalysis. *A*, model of the cyclooxygenase and peroxidase active sites of ovine PGHS-1. An alkyl hydroperoxide is shown bound to the heme group at the peroxidase active site, and arachidonate is shown bound to the cyclooxygenase active site. His-388 and His-207 are the proximal and distal heme ligands, respectively. Tyr-385 is likely the residue that abstracts the (13S)-hydrogen from arachidonate via a tyrosyl radical, thereby initiating cyclooxygenase catalysis. Ser-530 is the site of aspirin acetylation. Arg-120 is present at the opening of the hydrophobic substrate binding channel and is the countering for the carboxylate group of arachidonate. *B*, A model of PGHS-1 and PGHS-2 catalysis. A two-electron oxidation of the heme group of PGHS by a hydroperoxide yields a peroxidase spectral Intermediate I containing an oxyferryl form of iron (Fe(IV)) and a protoporphyrin radical cation. The oxidized heme group in turn oxidizes a neighboring tyrosine residue, probably Tyr-385 to yield peroxidase Intermediate II having a tyrosyl radical and an oxyferryl Fe(IV). This protein radical is likely the species that abstracts the (13S)-hydrogen from arachidonate. *PPIX, protoporphyrin IX; AA, arachidonic acid.*

and more accommodating than that of PGHS-1. Crystallography supports this view, and the relatively large compounds that specifically inhibit PGHS-2 take advantage of the space created by a valine to isoleucine substitution in PGHS-2 [35, 121].

Covalent modification of the enzymes is just one of three different inhibition mechanisms employed by common NSAIDS. Ibuprofen, the active ingredient in Advil® and Motrin®, inhibits the cyclooxygenase chemistry by simple reversible, competitive inhibition. Flurbiprofen and indomethacin are only slowly reversible; they are classified as time-dependent, reversible inhibitors. These drugs initially bind reversibly, but soon drive the formation of an *EI** complex from which their dissociation is very slow. Currently available NSAIDS inhibit both PGHS-1 and -2 with essentially equal potency.

When PGHS-2 was discovered it was almost immediately recognized by the pharmaceutical industry as a prime target to reduce pain and inflammation. A new group of NSAIDS are currently in clinical trials that were developed with the goal of inhibiting prostaglandin synthesis involved in inflammation while avoiding the gastrointestinal ulceration often associated with the inhibition of PGHS-1. These PGHS-2 specific inhibitors are all time-dependent, reversible inhibitors and have shown initial promise as non-ulcerogenic anti-inflammatory and anti-pyretic agents. Although their capacity to inhibit prostaglandin synthesis may be no better than that of common NSAIDS like aspirin, a significantly safer side-effect profile may allow PGHS-2 specific inhibitors to carve out an important clinical niche.

CHAPTER II

SUBCELLULAR LOCALIZATION OF PGHS-1 AND PGHS-2 BY IMMUNOELECTRON MICROSCOPY

Introduction

Prostaglandin endoperoxide H synthases-1 and -2 (PGHS-1 and -2) are the major targets of nonsteroidal anti-inflammatory drugs like aspirin and ibuprofen. These enzymes catalyze the committed step in the formation of prostanoids from arachidonic acid. Although PGHS-1 and -2 are similar biochemically, a number of studies suggest that PGHS-1 and PGHS-2 function independently to form prostanoids which subserve different cellular functions. We have hypothesized that these isozymes may reside, at least in part, in different subcellular compartments and that their compartmentation may affect their access to arachidonic acid and serve to separate the functions of the enzymes. To obtain high-resolution data on the subcellular locations of PGHS-1 and -2, we employed immunoelectron microscopy with multiple antibodies specific to each isozyme. Both PGHS-1 and -2 were found on the lumenal surfaces of the endoplasmic reticulum (ER) and nuclear envelope of human monocytes, murine NIH 3T3 cells, and human umbilical vein endothelial cells. Within the nuclear envelope, PGHS-1 and -2 were present on both the inner and outer nuclear membranes and in similar proportions. Western blotting data showed a similar distribution of PGHS-1 and -2 in subcellular fractions, and product analysis using isozyme-specific inhibitors suggested that both enzymes generate the same products in NIH 3T3 cells. Thus, we are unable to attribute the independent functioning of PGHS-1 and PGHS-2 to differences in their subcellular locations. Instead, the independent operation of these

isozymes may be attributable to subtle kinetic differences such that at low concentrations of arachidonate (ca. 50-1000 nM) this substrate is preferentially utilized by PGHS-2. A further conclusion of importance from a cell biological perspective is that membrane proteins such as PGHS-1 and -2, which are located on the lumenal surface of the ER, are able to diffuse freely among the ER and the inner and outer membranes of the nuclear envelope.

Methods

Materials. All materials were purchased from Sigma Chemical Company unless otherwise noted.

Isolation and preparation of human monocytes for electron microscopy. Peripheral blood mononuclear cells (PBMCs) were isolated from several healthy human volunteers. Heparinized blood was diluted 1:1 in Hanks Balanced Salts Solution (HBSS, GIBCO BRL, Grand Island, NY) and spun against a Ficol-Hypaque (LSM - Organon Teknika) gradient for 20 min at 500 x g. PBMCs were isolated as a band from the interface and washed 2 x with HBSS before resuspension in 10 ml of RPMI medium (GIBCO BRL, Grand Island, NY) containing 5% autologous serum and penicillin-streptomycin (100 U/ml). Cells (10^6/ml) were incubated in the presence or absence of 1 ng/ml of lipopolysaccharide (LPS; from Jayne Chen at Merck) and incubated with gentle agitation in a water-saturated 5% CO_2 atmosphere at 37 °C for 24 h. Fixation was performed after LPS- or vehicle (buffer alone)-stimulated cells were collected by centrifugation and resuspended in appropriate media (see below). Monocytes comprise 5-8% of PBMCs. Most of the other cells in PBMC preparations are T and B lymphocytes which do not normally express PGHSs and, therefore, provide a convenient internal negative control against PGHS immunostaining. Because ultrathin sectioning makes possible the observation of more than one section from a given cell, care was taken to use several different samples in the interest of gathering data from many different cells. PBMCs from each volunteer were separated into between five to ten separate pellets per experiment. Between every few grids of thin sections taken from a single pellet, several thick (1 μm) sections were cut to maximize the number of different cells observed per experiment.

Preparation of NIH 3T3 cells and HUVEC cells for electron microscopy. Murine NIH 3T3 cells express PGHS-1 constitutively [49]. Serum-starved, quiescent 3T3 cells were cultured and then stimulated by the addition of 10% fetal calf serum (HyClone Laboratories, Inc., Logan, UT) for 3 h to induce PGHS-2 as described previously [49]. After serum stimulation, cells from five 100 mm culture dishes were removed from the growing surface with a rubber policeman, resuspended in DMEM (GIBCO BRL, Grand Island, NY) and immediately fixed (see below). HUVECs (Cell Systems, Seattle, WA) were thawed from stocks frozen at passage 1 and expanded through passage 3 - 4 as suggested by the manufacturer. After stimulation for 20 h with 10 ng/ml IL-1β, cells were removed from culture dishes with a rubber policeman, immediately resuspended in CS-C media (Cell Systems, Seattle, WA) and fixed (see below). Previous PGHS immunolocalization experiments on both adherant and removed cells gave similar results, suggesting that removal of cells from the growing surface does not affect the localization of PGHS-1 or PGHS-2 [28, 67].

Fixation and cryoprotection for electron microscopy. Cells (PBMCs, NIH 3T3, HUVEC) were obtained in a 2.5 ml suspension of an appropriate medium and immediately fixed by the addition of 2.5 ml of 2X fixative. Two different fixation methods were employed to allow for differences in the fixation sensitivity of PGHS-1 and -2 antigens. Microwave/glutaraldehyde fixed cells were fixed exactly as described previously [69]. Cells fixed with Nakane fixative [122] alone were treated by adding 2X Nakane fixative (1X Nakane = 0.1 M $NaIO_4$, 0.75 M Lysine, 0.0375 M phosphate buffer, 2% paraformaldehyde (Fisher)) to an equal volume of cell suspension and incubated for two h at room temperature.

These cells were then washed twice with 1X Nakane fixative and resuspended in fresh 1X Nakane before an overnight (or shorter) incubation at 4 °C. NIH 3T3 cells immunolabeled for PGHS-2 were only fixed at 4 °C for an additional 2 h after the 2 h room temperature fixation. After the overnight fixation step, both microwave/glutaraldehyde- and Nakane-fixed cells were collected by centrifugation, resuspended in 0.1 M sucrose/PBS, and pelleted in 2% low gelling temperature agarose/0.1 M sucrose/PBS. Cryoprotection was performed for 2 h (25 °C) or overnight (4 °C) in polyvinylpyrrolidone/2.3 M sucrose/phosphate buffer pH 7.2 (PVP-sucrose) [69]. Cell pellets were mounted on bullseye specimen pins (Ted Pella, Inc.), frozen by plunging into liquid propane, and stored under liquid nitrogen until use.

Immunogold labeling. Immunogold labeling was performed essentially as described previously [69]. Briefly, ultrathin (75-80 nm) sections of cells were cut at -106 °C on a Reichert Ultracut S ultramicrotome fitted with a Reichert FCS cryoattachment. Sections were collected on drops of 2.3 M sucrose and placed on glow discharged, formvar-coated nickel grids (Ted Pella, Inc.). After a minimum of one h in blocking solution (5% milk/1% BSA/PBS/0.02% sodium azide) and washing, grids were placed section side down on 25 µl drops of primary antibody solutions of various concentrations and incubated for various times depending on the primary antibody. All antibody solutions were cleaned by filtration through 0.2 µm filters prior to use. To control for antibody specificity, primary antibodies were incubated with a 50-fold molar excess of either cognate peptide (for anti-PGHS-1 or anti-PGHS-2 peptide directed antibodies) or an approximately 10-fold molar excess of purified ovine PGHS-1 or -2 (for antibodies prepared against either whole protein). Preadsorption of whole protein antibodies with purified PGHS-1 or -2 was performed with

constant agitation at 4 °C for 24 h. The samples were centrifuged at 12,000 rpm in a Beckman microcentrifuge for 1 h at 4 °C, the supernatants were removed, filtered through a 0.2 μm syringe filter, and used as primary antibody solutions in immunogold labeling experiments. Purified ovine PGHS-1 was from R.M. Garavito at Michigan State University; purified ovine PGHS-2 was from Cayman Chemical Co., Ann Arbor, MI. After incubation with primary antibody, each grid was washed eight times for three min on drops of 1% BSA/PBS/0.02% sodium azide before a 1 h incubation at room temperature on drops of gold-conjugated secondary antibody (Amersham GAR-G5 diluted 1:75 in 1% BSA/PBS/0.02% sodium azide). After a washing step, sections were post-fixed and stained for contrast by floating the grids sequentially on drops of 2% glutaraldehyde, 2% osmium tetroxide, and 2% uranyl acetate. Polyvinyl alcohol (2%) was used to embed the grids before observation by transmission electron microscopy [123].

Antibodies specific for PGHS-1 and PGHS-2. All primary antibodies were raised in rabbits as described previously [42]. For PGHS-2 staining of human monocytes and HUVECs, a polyclonal antibody raised against ovine PGHS-2 was used. This antibody (from Dr. Jilly Evans, Merck Frosst) cross-reacts with human PGHS-2 but not with ovine or human PGHS-1 [124]. PGHS-1 immunostaining of NIH 3T3 mouse fibroblasts was performed with an affinity purified anti-peptide antibody directed against amino acids Leu274 - Arg288 of murine PGHS-1 [125]. This antibody does not cross react with PGHS-2 in Western blotting experiments. Another antibody, an IgG fraction from a polyclonal antibody raised against PGHS-1 [126], recognizes only PGHS-1 and was used for immunolabeling of NIH 3T3 cells and HUVECs. PGHS-2 labeling of NIH 3T3 cells was

performed using an affinity purified anti-peptide antibody directed against an 18 amino acid cassette near the C-terminus of PGHS-2 [67, 127]; this antibody does not cross react with any known PGHS-1 but recognizes human and murine PGHS-2 on Western blots.

Distribution of PGHS-1 and PGHS-2 between inner and outer membranes of the nuclear envelope. We determined the distribution of PGHS-1 and PGHS-2 between the inner and outer nuclear membranes essentially as described previously [69]. Gold particles lying on or within one 5 nm particle diameter of the inner nuclear membrane were designated as being on the inner nuclear membrane. Particles lying on or within one 5 nm particle diameter of the outer nuclear membrane were designated as being on the outer nuclear membrane. When a gold particle was observed in the nuclear envelope and not within one particle diameter of either membrane, it was designated as lumenal. Analysis of the distribution of PGHS-1 and PGHS-2 was performed on a total of 24 and 32 cells, respectively. Distribution analysis was limited to *well-preserved* sections of nuclear envelope. Only regions of cells in which the inner and outer nuclear envelope were clearly distinguishable were used for our analyses.

Statistical Analysis. The distribution analysis described above was performed using at least three experimental groups of well-preserved cells for both PGHS-1 in NIH 3T3 cells and PGHS-2 in monocytes. The analysis of PGHS-2 in NIH 3T3 cells was performed on seven cells taken from two separate experiments. Analysis of the mean distribution percentages using Student's *t* test showed that there was no significant difference between the inner membrane/outer membrane distribution of PGHS-1 and -2 in the cell types analyzed. It is possible for a protein of the nuclear envelope to be present in significantly

different amounts, as evidenced by previous work on 5-lipoxygenase and FLAP [69].

Subcellular fractionation and Western analysis. NIH 3T3 cells were prepared as whole cell lysates, microsomes, or isolated nuclei. For whole cells, harvests of three 100 mm culture dishes of serum-stimulated NIH 3T3 cells in PBS were collected by centrifugation and resuspended in Hanks Balanced Salts Solution (HBSS). The resuspended cells were then sonicated and dounce homogenized to produce whole cell lysates. Microsomes were prepared as described previously [27, 117, 128] from ten plates of cells except that the 200,000 x g pellet was resuspended in HBSS. Isolation of cell nuclei [129, 130] began with the harvest of twenty dishes of 3T3 cells in PBS followed by centrifugation at 1000 x g. Nuclei were isolated in the presence of 0.2% or 1% saponin as described below. Isolation of nuclei in the absence of detergent was begun by resuspension in 10 ml of cold Buffer A (10 mM Tris, 10 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 1 μg leupeptin/ml, and 3 mM $MgCl_2$, pH 8.0) and incubation on ice for 5 min, followed by 5 seconds of gentle vortexing. After passage ten times through a 20-gauge needle, the crude nuclei were again collected by centrifugation at 1000 x g. The pellet was resuspended in 6 ml of cold Buffer A and incubated on ice for 5 min. After an additional ten passages through a 20-gauge needle, the suspension was subjected to gentle homogenization in a Teflon homogenizer. Half of the suspension was placed in each of two Beckman 5 ml UltraClear ultracentrifuge tubes. A nuclear spin cushion was prepared by dissolving 6.16 g sucrose in 10 ml of Buffer B (60 mM KCl, 15 mM NaCl, 15 mM Tris, 0.15 mM spermine, 0.5 mM spermidine, and 0.5 mM DTT) to give a 1.8 M sucrose solution. After underlaying 2 ml of the sucrose solution beneath the crude nuclear suspension, the tubes were centrifuged for 19 min at 4 °C at 13,500

rpm in a Beckman SW50.1 rotor. Isolated nuclei were visible at the bottom of the tubes. The liquid was removed from the tubes by aspiration, and the nuclear pellet was resuspended in 400 µl of HBSS. Nuclei were washed 4 times with HBSS and collected by centrifugation at 1800 rpm for three min in a microcentrifuge. Purified nuclei and whole cell lysates were disrupted by sonication before protein concentrations were determined using a BioRad Protein Assay solution. Aliquots of broken cells, microsomes, or nuclei (20 µg) were separated by SDS-PAGE and analyzed by Western blotting as described previously [41]. Densitometric quantitation of immunoreactive PGHS-1 or -2 was performed using a BioRad GS-505 Molecular Imaging System and Molecular Analyst software. For a given isozyme, densities are expressed as the ratio of nuclear to microsomal immunoreactivity. Student's t test was utilized to determine if these ratios for PGHS-1 and PGHS-2 were significantly different for each experimental condition.

Results

Immunogold labeling of PGHS-2 in human monocytes and HUVECs. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human volunteers, treated for 20 h with bacterial lipopolysaccharide (LPS), and processed for electron microscopy. Western blotting of samples of these cells prior to fixation established that, as expected, PGHS-2 expression was induced by LPS, but that vehicle-treated cells lacked detectable levels of this isozyme [131]. Monocytes in LPS-treated samples, when incubated with an antibody raised against ovine PGHS-2, exhibited perinuclear staining for PGHS-2 (Fig. 10A). PGHS-2 labeling was completely eliminated by preadsorption of the anti-PGHS-2 antibody with purified ovine PGHS-2 (Figure 10B). No comparable gold label was seen in vehicle-treated cells (data not shown). PGHS-2 labeling was observed on both the inner and outer membranes of the nuclear envelope in monocytes present in LPS-treated PBMC preparations (Fig. 10C). The distribution of PGHS-2 within the nuclear envelope of 26 LPS-treated monocytes was determined by counting the gold particles associated with well-preserved regions of the inner and outer nuclear membranes (Table I). In performing these distribution analyses, only those segments of the nuclear envelope in which both the inner and outer nuclear membranes were clearly distinguishable were used. PGHS-2 labeling was approximately equally distributed between the inner and outer membranes of the nuclear envelope.

To determine if the distribution pattern of PGHS-2 was the same in cell types other than monocytes, we performed additional studies with (a) serum treated murine NIH 3T3 cells and (b) IL-12-treated human umbilical vein endothelial cells (HUVEC) both of which are known to express PGHS-2 [49, 132]. An anti-peptide antibody raised against the

Figure 10. Immunogold labeling of PGHS-2 in human monocytes. Human peripheral blood mononuclear cells were isolated, fixed and processed for electron microscopy as detailed in the text. Ultrathin cryosections were labeled with either a primary antibody specific for PGHS-2 (A,C) or a primary antibody specific for PGHS-2 which had been preadsorped with purified PGHS-2 (B). This was followed by treatment with a secondary anti-rabbit IgG-gold conjugate. (A, B) Sections of monocytes showing a portion of the nucleus and cytoplasm with gold label denoted by small arrowheads. (C) region of a nuclear envelope with small arrowheads denoting gold particles on the outer (*o*) nuclear membrane and large arrowheads denoting label on the inner (*i*) nuclear membrane. In (C), opposed, open arrows indicate well-preserved sections of nuclear envelope representative of sections used in the analysis of PGHS-2 distribution between the inner and outer nuclear membranes. N, nucleus; er, endoplasmic reticulum. Scale bar = 0.1 μm.

er
N
er
A
N
B
o
i
N
C

Isozyme (Cell type)	Total Gold Particles			Percentage of Total Gold Particles (%)		
	Inner	Outer	Lumenal	Inner	Outer	Lumenal
PGHS-2 (monocyte)	123	165	52	37±2	49±2	15±3
PGHS-2 (3T3 cells)	27	20	8	47±7	39±6	14±3
PGHS-1 (3T3 cells)	82	83	34	41±2	43±4	17±3

Table I. Analysis of PGHS-1 and PGHS-2 distribution within the nuclear envelope. The distributions of PGHS-1 and PGHS-2 within the nuclear envelope of LPS-treated human monocytes and serum-treated murine NIH 3T3 cells were determined as detailed in Materials and Methods. In this analysis gold particles were counted as being associated with either the *inner* or *outer* nuclear membrane if they were touching or within one particle diameter of that membrane. Other particles, not within one particle diameter of either membrane, were designated here as *lumenal*. Only well-preserved regions of nuclear envelopes (i.e. regions in which both the inner and outer nuclear membranes were visible) were used for this analysis. For monocytes, particles representing PGHS-2 from a total of 26 cells were counted. For NIH 3T3 cells, a total of 27 and 7 cells were analyzed for particles corresponding to PGHS-1 and PGHS-2, respectively. Analysis of the mean distribution percentages using Student's *t* test showed that there was no significant difference between the inner membrane/outer membrane distribution of PGHS-1 and -2 in the cell types analyzed.

C-terminal 18 amino acid cassette of PGHS-2 was used to label PGHS-2 in serum-treated NIH 3T3 cells. This antibody labeled serum-treated NIH 3T3 cells along the nuclear membrane and in the ER (not shown). PGHS-2 immunoreactivity was distributed equally between the inner and outer nuclear membranes in NIH 3T3 cells (Table I). PGHS-2 labeling of serum-treated NIH 3T3 cells was eliminated by preincubation of the antibody with its cognate peptide. Finally, PGHS-2 staining in IL-1β- treated HUVECs was present on the inner and outer nuclear membranes and in the ER, a pattern similar to that seen in LPS-treated monocytes and serum-treated NIH 3T3 cells (data not shown).

Immunogold labeling of PGHS-1 in murine NIH 3T3 cells. Serum-starved or serum-treated NIH 3T3 cells were fixed and processed for immunoelectron microscopy. The expectation that PGHS-1 would be expressed at similar levels in both starved and treated cells [49, 133] was confirmed by western blotting (not shown). Serum-treated (Fig. 11A) or serum-starved (not shown) NIH 3T3 cells exhibited perinuclear and ER staining for PGHS-1. PGHS-1 labeling was eliminated by preincubation of the anti-PGHS-1 antibody with its cognate peptide (Fig. 11B). The immunostaining experiments depicted in Fig. 11 were performed using an anti-PGHS-1 antibody raised against a peptide corresponding to amino acids Leu274-Arg288 of murine PGHS-1. Identical experiments performed with another anti-PGHS-1 antibody, this one raised against whole ovine PGHS-1 [126], resulted in a pattern of staining indistinguishable from that seen with the anti-peptide antibody (not shown). PGHS-1 staining by the antibody prepared against the whole protein was eliminated by preadsorption of the antibody with purified ovine PGHS-1. Several experiments were performed using a total of 24 different NIH 3T3 cells in which well-preserved sections

Figure 11. Immunogold labeling of PGHS-1 in murine NIH 3T3 cells. NIH 3T3 cells were serum-treated, fixed and processed for electron microscopy as detailed in the text. Ultrathin cryosections were labeled with either an affinity purified rabbit anti-peptide antibody to the sequence Leu274-Arg288 of murine PGHS-1 (A, C) or the same antibody preincubated with its cognate peptide (B). This was followed by treatment with a secondary anti-rabbit IgG-gold conjugate. (A, B) Sections of NIH 3T3 cells showing portions of the nucleus and cytoplasm with gold label denoted by small arrowheads. (C) Region of a nuclear envelope with small arrowheads denoting gold particles on the outer (*o*) nuclear membrane and large arrowheads denoting label on the inner (*i*) nuclear membrane. In (C), opposed, open arrows indicate well-preserved sections of nuclear envelope representative of sections used in the analysis of PGHS-1 distribution between the inner and outer nuclear membranes. Nu, nucleus; er, endoplasmic reticulum. Scale bar = 0.1 μm.

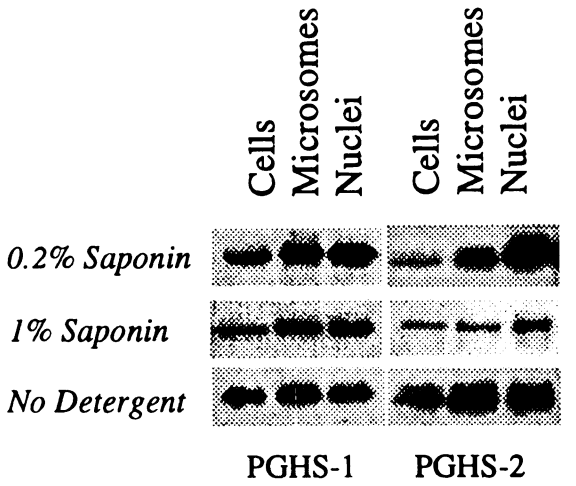
A
Nu
B
Nu
C
Nu

of the nuclear envelope were analyzed to determine the distribution of PGHS-1 between the inner and outer nuclear membranes (Fig. 11C). Gold particles representing PGHS-1 staining along the NE were present in approximately equal abundance on the inner and outer nuclear membranes (Table I). Our results confirm that PGHS-1 is a membrane protein of the ER and nuclear envelope [28] and establish that PGHS-1 is distributed equally between the membranes of the nuclear envelope. Collectively, the data demonstrate that PGHS-1 and -2 reside in the same subcellular membranes.

Western blotting of PGHS-1 and PGHS-2 in subcellular fractions. Earlier immunofluorescence studies from our laboratory indicated that PGHS-1 and PGHS-2 are in the ER and nuclear envelope but that the concentration of PGHS-2 in the nuclear envelope is roughly twice that of the ER while the concentration of PGHS-1 is the same in the nuclear envelope and the ER [67]. In contrast, our quantitative immunoelectron microscopy indicated that PGHS-1 and -2 are present in the same subcellular locations in approximately equal proportions. There are significant differences between the fixation and staining protocols used for immunofluorescence and electron microscopy any one of which could conceivably cause subtle differences in the patterns of staining. One difference involves the use of detergents to permeabilize the cells for immunofluorescence staining. Accordingly, we determined the effect of saponin, the detergent used in our earlier immunofluorescence work [67], on the distribution of PGHS-1 and PGHS-2 in nuclear and ER fractions from NIH 3T3 cells. When nuclei were isolated in the presence of 1% saponin from murine NIH 3T3 cells, immunoreactive PGHS-2 was relatively more abundant in nuclear membranes than in microsomal membranes while PGHS-1 immunoreactivity was equally distributed between

nuclear and microsomal membranes in the same experiments (Fig. 12). Similar results were obtained when nuclei were isolated in the presence of 0.2% saponin, although the difference between PGHS-1 and PGHS-2 distribution were not statistically significant. However, when Western blotting experiments were conducted on subcellular fractions isolated from serum-stimulated murine NIH 3T3 cells in the absence of detergent (Fig. 12), PGHS-1 and PGHS-2 were similarly distributed between nuclear and microsomal membranes. We measured the ratios of immunoreactive PGHS-1 and -2 in nuclei versus microsomes using densitometry. In three experiments in the presence of saponin, the ratios of nuclear to microsomal immunoreactive PGHS-1 and -2 were 1.1 +/- 0.1 and 1.9 +/- 0.2, respectively. In the presence of 0.2% saponin, the same ratios for PGHS-1 and-2 were 1.14 +/- 0.1 and 1.26 +/- 0.1. Exclusion of detergent resulted in nuclear to microsomal immunoreactivity ratios for PGHS-1 and -2 of 1.08 +/- 0.1 and 1.10 +/- 0.2, respectively. These data suggest that PGHS-1 and PGHS-2 can be differentially solubilized from nuclear membranes by high concentrations of detergents such as saponin. This may account for the differential patterns of PGHS-1 and PGHS-2 staining that have been observed in immunofluorescence studies [67].

A.



B.

	Ratio of nuclei:microsomes	
0.2% Saponin	1.14 +/- 0.06	1.26 +/- 0.06
1% Saponin	1.1 +/- 0.1	1.9 +/- 0.2
No Detergent	1.10 +/- 0.20	1.08 +/- 0.14
	PGHS-1	PGHS-2

Figure 12. Western blot analysis of PGHS-1 and PGHS-2 in subcellular fractions of murine NIH 3T3 cells. Whole cells, microsomes or nuclei were prepared from murine NIH 3T3 cells in the presence of 0.2% saponin, 1% saponin, or no detergent as described in the text. *A*, After separation of the protein fractions by SDS-PAGE and transfer to nitrocellulose, isozyme-specific antibodies were used as indicated to visualize PGHS-1 and PGHS-2 immunoreactivity in each membrane fraction. *B*, Densitometric analysis was used to determine the ratio of immunoreactive PGHS-1 or PGHS-2 in nuclear versus microsomal membranes. Ratios obtained using 1% saponin were significantly different from each other according to Student's *t* test.

Discussion

PGHS-1 and PGHS-2 have structurally homologous membrane binding domains containing four amphipathic helices which anchor the proteins to one leaflet of the lipid bilayer [28, 34, 35, 131, 134-136]. Previous work has established that both isozymes are located on the lumenal surfaces of the ER and nuclear envelope [28, 42]. However, immunocytofluorescence studies suggested that PGHS-2 was more concentrated in the nuclear envelope than the ER whereas PGHS-1 was equally distributed in both compartments [67]. One possibility to account for these latter findings was that PGHS-2 was uniquely localized on the inner membrane of the nuclear envelope. The major aim of the present studies was to examine this possibility using immunoelectron microscopy. The issue of PGHS-1 versus PGHS-2 localization is of particular interest because of the potential relationship between PGHS-2-derived products generated at the nuclear envelope and nuclear signaling associated with cell replication or differentiation.

The major finding of our EM study is that in NIH 3T3 cells both PGHS-1 and PGHS-2 are present in equal proportions on both the inner and outer membranes of the nuclear envelope. Human monocytes and umbilical vein endothelial cells stained for PGHS-1 or PGHS-2 exhibited identical patterns. Our results make it clear that both isozymes are present in the same subcellular compartments and at comparable concentrations. Of course, we cannot rule out the possibility that PGHS-1 and PGHS-2 are associated with different microdomains within these compartments.

Earlier results from immunofluorescence studies which employed low concentrations of saponin to permeabilize cell membranes and had suggested that PGHS-2 is preferentially localized to the nuclear envelope [67] can be explained on the basis of differential

solubilization of the two proteins from the nuclear envelope. When ER and nuclear membranes were prepared in the presence of 1% saponin, PGHS-2 was more concentrated in the nuclear fraction while PGHS-1 was found in equal abundance in the ER and nuclear membranes; in the absence of saponin, PGHS-2 was present at the same concentration in the ER and nuclear envelope. Presumably these differences in affinities of the two proteins for the nuclear envelope are a result of the significant differences in the amino acid composition of the membrane binding domains of the two isozymes [1, 43].

A model has emerged suggesting that PGHS-1 and PGHS-2 act independently and that, at least in part, the inducible isozyme, PGHS-2, provides prostaglandins for a nuclear eicosanoid signaling system [1, 104, 137]. While this model may still be correct, our results imply that any specific connection between PGHS-2 and the generation of products which function in the nucleus would have to result from differences in the expression of the activities of PGHS-1 and PGHS-2 and not from gross differences in the subcellular distributions of PGHS-2 versus PGHS-1. That is, because both PGHS-1 and PGHS-2 appear to be present in the same membranes, factors other than compartmentation account for the separation of their activities into two independent systems. The most likely factors are differences in interactions with different phospholipases and/or differences in enzyme kinetics. Arm, Austen and Herschman and their colleagues [78, 79, 138-140] have demonstrated that two separate phases of PGD_2 synthesis in mast cells are independently coupled to PGHS-1 (early phase) and PGHS-2 (late phase) by different phospholipases A_2. Kinetic mechanisms for separating the actions of PGHS-1 and PGHS-2 have also been described. For example, PGHS-2 has a significantly lower threshold for hydroperoxide

activation than PGHS-1 thereby enabling PGHS-2 to oxygenate arachidonic acid in the presence of lower peroxide concentrations [141, 142]. In addition, negative allosteric regulation of PGHS-1 by arachidonic acid, at concentrations between 0.5 nM and 1 μM, has the overall effect of causing a 2-4 fold greater rate of PGHS-2-mediated prostanoid formation [143]. These kinetic differences between PGHS-1 and PGHS-2 have been identified with purified or partially purified enzyme preparations. However, it may be possible to test for kinetic differences between the two isozymes in intact cells using histochemical assays of enzyme activity [67].

Another more speculative possibility to account for the independent operation of PGHS-1 and PGHS-2 in cells where both isozymes are expressed is the existence of accessory proteins which differentially affect the rate of prostaglandin endoperoxide formation by PGHS-1 versus PGHS-2. Although no such protein(s) has been identified in the prostanoid biosynthetic system, there is a precedent for an accessory protein in the leukotriene pathway. Leukotrienes synthesized through 5-lipoxygenase arise from arachidonic acid apparently delivered to the 5-lipoxygenase by an activating protein, FLAP [85, 143-147].

The observation that both PGHS-1 and PGHS-2 are located on the inner nuclear membrane is of interest from a cell biology perspective. To our knowledge, no other endogenous integral membrane proteins of the ER have been demonstrated to be present on the inner nuclear membrane. FLAP, another integral membrane protein involved in eicosanoid biosynthesis has been localized to the inner and outer membranes of the NE [69, 70]. However, in contrast to PGHS-1 and -2, FLAP is predominantly localized to the nuclear

envelope; the orientation of FLAP in the membrane is not well characterized. One model of how integral membrane proteins synthesized in the ER reach the inner nuclear membrane involves lateral diffusion through the membrane bilayers of the NE [69, 70, 114, 115, 148]. According to this model, membrane proteins are subject to a size constraint imposed by the lateral channel diameter of the nuclear pore complex, which serves to separate the inner and outer nuclear membranes. Both PGHS-1 and PGHS-2 are targeted initially to the ER by a KDEL-like C-terminal targeting signal [149]. We propose that both isozymes are then able to bypass the nuclear pore complex and reach the inner nuclear membrane by lateral diffusion. Our reasoning is based on the nature of their interaction of PGHS-1 and PGHS-2 with membranes (i.e. via a monotopic membrane binding domain) and the fact that the proteins are on the lumenal surface of the membrane (Fig. 13). Consistent with this concept are studies with the lamin B receptor (LBR), an integral membrane protein that is targeted exclusively to the inner nuclear membrane via specific targeting signals [114]. When the nucleoplasmic/cytoplasmic-oriented extramembrane domain of LBR is artificially enlarged, the protein is retained in the ER (and outer nuclear membrane). Other ER membrane proteins with large extramembrane domains oriented towards the cytoplasm are also thought to be restricted to the outer membrane of the nuclear envelope and the ER [114, 150]. P450s such as thromboxane synthase and prostacyclin synthase are integral membrane proteins of the ER and have relatively large cytoplasmic domains. Thus, these proteins are not likely to be present on the nucleoplasmic face of the inner nuclear membrane and would be unable to metabolize efficiently PGH_2 generated by PGHS-1 or PGHS-2 present on this membrane.

We conclude that PGHS-1 and PGHS-2 are present in similar proportions on the endoplasmic reticulum, outer nuclear membrane, and inner nuclear membrane of NIH 3T3 cells, human monocytes, and HUVECs. Fatty acid substrates for PGHSs appear to be supplied via both an $sPLA_2$ that functions on the phospholipids on the cell surface and a $cPLA_2$ that undergoes a Ca^{2+}-dependent translocation to the cytosolic face of the ER and outer membrane of the nuclear envelope [59, 61, 65, 151, 152] and perhaps the inner membrane of the nuclear envelope [66]. However, the issue of whether there is preferential coupling of different phospholipases A_2 to PGHS-1 versus PGHS-2 is currently unresolved. PGH_2 formed through both PGHS-1 and PGHS-2 can apparently diffuse readily through membranes [153]. That PGH_2 which diffuses from the ER lumen into the cytoplasm is likely to be metabolized by enzymes such as TxA synthase or PGI_2 synthase located on the cytoplasmic surface of the ER and nuclear envelope [154]. The fate of the PGH_2 which diffuses into the nucleoplasm is presently unknown.

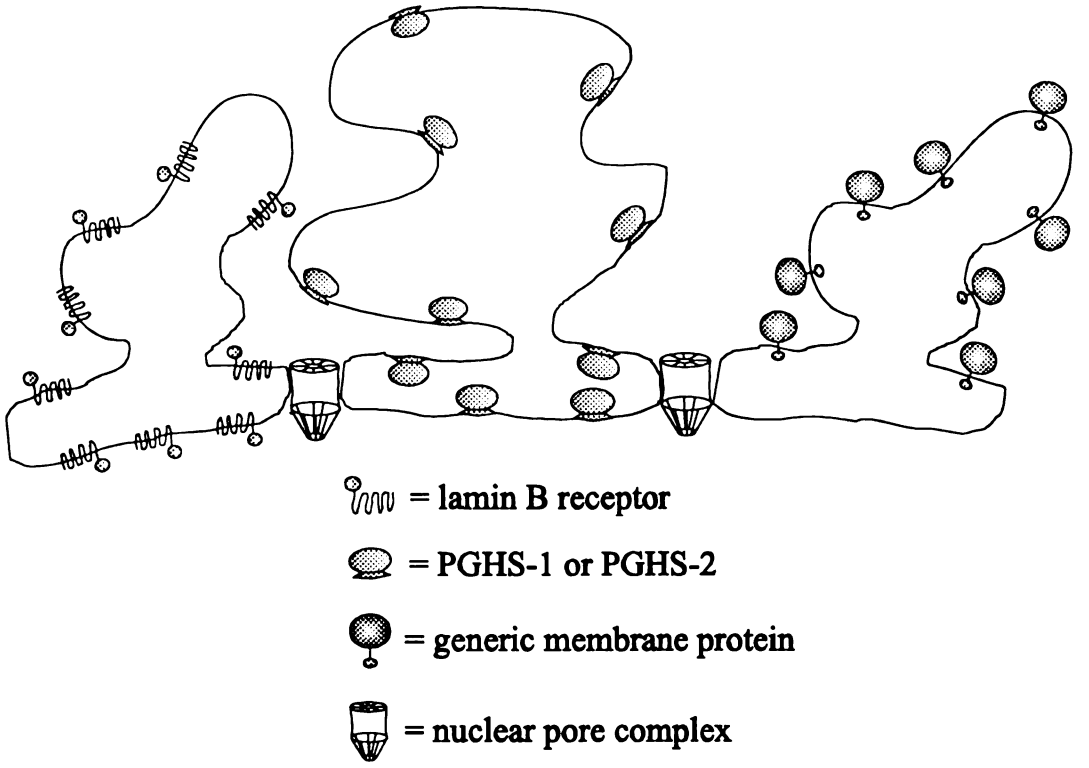


Figure 13. Diffusion of integral membrane proteins in the nuclear envelope. Membrane topography of integral membrane proteins affects their ability to migrate through the nuclear envelope [114]. The inner and outer nuclear membranes are biochemically and functionally distinct, and are physically separated by the nuclear pore membranes and pore complexes (NPC). Lumenally-oriented proteins like PGHS-1 and -2 are able to reach the inner nuclear membrane presumably by passive diffusion. Likewise, the cytoplasmically-oriented lamin B receptor, which is found exclusively on the inner nuclear membrane, has a small extramembrane domain that is not held up at the NPC. One can speculate that membrane proteins with large cytoplasmic domains will be physically prevented from reaching the inner nuclear membrane due to the barrier of the NPC.

Acknowledgment

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CHAPTER III

THE MEMBRANE ASSOCIATION OF PGHS-1 AND PGHS-2

Introduction

PGHS-1 and PGHS-2 are integral membrane proteins by definition since detergents, and not just chaotropic salts, are required to disrupt their association with cellular membranes [18]. X-ray crystallographic studies have demonstrated that neither isozyme contains structural motifs that are well-known to confer membrane association to integral membrane proteins (e.g., transmembrane domains or prenylation sites). Instead, PGHS-1 and -2 are thought to associate with cellular membranes through a novel, monotopic membrane binding domain that traverses a single leaflet of the lipid bilayer.

Blobel speculated in 1980 on the existence of monotopic integral membrane proteins [155], but the first structural evidence for such a protein came from the X-ray crystallographic description of ovine PGHS-1 by Picot, et al. [34]. The tertiary structure of PGHS-1 is predominantly globular. Projecting from the globular catalytic domain in each monomer are four amphipathic alpha helices (labeled A, B, C, and D in Fig. 6). Hydrophobic and aromatic residues protrude from these helices and away from the hydrophilic surface of the catalytic domain to create a hydrophobic patch. These helices also form the opening to the cyclooxygenase active site and were predicted by Picot et al. to facilitate interaction with the lipid bilayer. It is important to our understanding of PGHS biology to know the mechanism by which PGHS-1 and -2 interact with cellular membranes. The substrate of PGHSs, arachidonic acid, is released from the bilayer and presumably

travels through the core of helices A-D en route to the cyclooxygenase active site. As discussed in Chapter II, the topology of membrane proteins plays a significant role in their subcellular localization. In addition, a thorough description of the membrane binding domains of PGHS-1 and -2 may provide new information regarding a recently-discovered membrane association strategy that may be used by other lipid biosynthetic enzymes.

We have aimed to characterize biochemically the regions of PGHS -1 and PGHS-2 that interact with cellular membranes using 3-trifluoro-3-(m-[^{125}I]iodophenyl)diazirine ([^{125}I]-TID). [^{125}I]-TID has been used as a tool to selectively label the hydrophobic regions of integral membrane proteins [156, 157]. The compound is hydrophobic, photoactivatable, and partitions into lipid bilayers where, following activation with UV light, it cross-links to membrane-associated regions of proteins (Fig. 14). In 1996, Otto and Smith used [^{125}I]-TID to show that an N-terminal region of ovine PGHS-1 associates with membranes between amino acids 25-166 [135]. Here, we extend this work and provide similar evidence in PGHS-2 that the putative membrane binding domain predicted by Picot et al. is indeed associated with membranes in the endoplasmic reticulum.

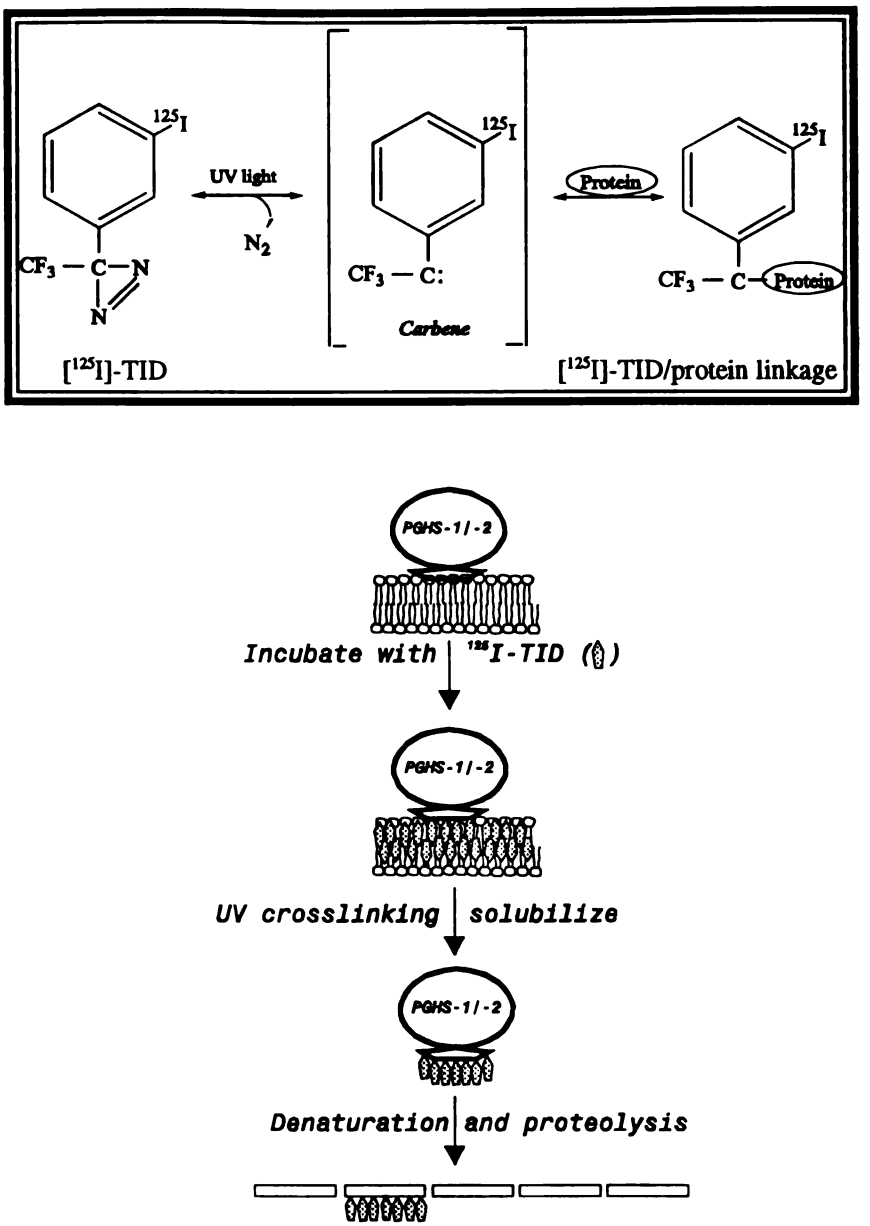

Figure 14. Mechanism of [^{125}I]-TID cross linking to membrane proteins. [^{125}I]-TID is a hydrophobic, photoactivatable compound that can be used to label the membrane-associated regions of proteins. Due to its extreme hydrophobicity, [^{125}I]-TID labels only hydrophobic regions of membrane proteins and does not label regions of proteins exposed to an aqueous environment [156]. *Top:* Photoactivated release of the diazirine group of [^{125}I]-TID as dinitrogen leads to the formation of a highly reactive carbene that nonspecifically cross-links to constituents of cellular membranes, including both proteins and lipids. This chemistry allows one to covalently label the membrane-associated region of a protein with a radioactive marker. *Bottom:* Schematic diagram of a [^{125}I]-TID labeling experiment.

Materials and Methods

Preparation of PGHS-1 from ovine seminal vesicles. Microsomal ovine PGHS-1(oPGHS-1) was obtained from sheep seminal vesicles essentially as described previously [135]. Briefly, 5 g of seminal vesicles were sliced into 1 mm strips with a razor blade and suspended in 30 ml HEPES buffer (20 mM HEPES, 20 mM glutamic acid, 2 mm magnesium acetate, 200 mM sucrose, pH 7.5). After three 30 second blasts with a Polytron homogenizer, heavy tissue debris was pelleted by centrifugation at 10,000 x *g*. Microsomal membranes in the supernatant of the low speed spin were collected by ultracentrifugation at 200,000 x *g* in a Beckman SW50.1 rotor. The microsomal pellet was resuspended in 2 ml HEPES buffer, dounce homogenized, cleared by centrifugation at 10,000 x g, and assayed for protein using the BioRad Bradford protein assay reagent.

Preparation of PGHS-2 from baculovirus-infected Sf21 cells. Sf21 insect cells were grown in 500 ml spinner flasks or a 12 L bioreactor in Sigma TC-100 medium containing 10% fetal calf serum. When the cell density reached 1.8 x 10^6 cells/ml, cells were infected with baculovirus containing a gene for human PGHS-2 (hPGHS-2) that had been engineered to contain a hexa-Histidine tag at its N-terminus. Infection was allowed to proceed for three days. Approximately 9.0 x 10^8 Sf21 cells were harvested by centrifugation and resuspended in 10 ml 0.1 M Tris, pH 7.4. Cells were then disrupted by vigorous sonication using a microtip attachment to a Misonix Sonicator. After a low speed (10,000 x g) spin to pellet cell debris, microsomal membranes were collected by centrifugation at 200,000 x g in a Beckman SW50.1 swinging bucket rotor. The microsomal pellet was resuspended in 2 ml 0.1 M Tris, pH 7.4, dounce homogenized, and assayed for protein using the BioRad Bradford

protein assay reagent.

[^{125}I]-TID labeling of PGHS-1 and -2. This procedure was carried out at 4°C unless otherwise noted. Microsomal preparations of oPGHS-1 or hPGHS-2 were prepared as described above and resuspended to a final protein concentration of 15-20 mg/ml in the appropriate buffer. Aliquots (400 ul) were preincubated in the presence of 80 μM flurbiprofen to block the ability of [^{125}I]-TID to label the PGHS active site. In some experiments, 200 mM reduced glutathione was included to scavenge [^{125}I]-TID exposed to the aqueous phase. [^{125}I]-TID was added to a final concentration of approximately 5 μM. After incubation at 4°C for 10 min., resuspended microsomes were transferred to a 1 cm diameter plastic culture dish and irradiated for 10 min. with a UV illuminator (366 nm) held at a distance of 5 cm. Labeled membrane proteins were then solubilized from cellular membranes using the detergent $C_{10}E_6$ (Anatrace, Inc.) at a concentration of 1% (v/v) for 1 hour with gentle shaking. oPGHS-1 or hPGHS-2 were purified from the solubilized membrane protein fraction using immunoprecipitation or Ni-Agarose, respectively.

Immunoprecipitation of PGHS-1 from solubilized microsomes. Following treatment with [^{125}I]-TID, solubilized microsomal membrane proteins were transferred to a new tube. A monoclonal antibody directed against oPGHS-1 (50 μl, concentration: 0.1 mg/ml) [158] was added to the solubilized proteins and the sample allowed to incubate with shaking for 30 min at 4°C. PGHS-1 was precipitated by the addition of 100 μl of Protein A-Sepharose (50% slurry in 0.1 M Tris/0.1% Tween 20/pH 8.0) and incubation for 30 min. at 4°C. The immunocomplex was pelleted at 500 x g in a tabletop microcentrifuge and washed three times with 0.1 M Tris/0.1% Tween 20/pH 8.0. To elute immunoprecipitated oPGHS-1, the

immunocomplex was resuspended in 0.5% recrystallized SDS and boiled for 5 min with periodic vortexing. The Protein A-Sepharose was then pelleted at high speed, and the supernatant was removed. The supernatant containing the immunopurified, [^{125}I]-TID labeled oPGHS-1 was then (a) subjected to immediate proteolysis or (b) denatured, reduced, and alkylated prior to proteolysis. Immediate proteolysis required the dilution of 0.5% SDS to 0.05% SDS by diluting 10-fold in an appropriate buffer and reconcentrating the [^{125}I]-TID labeled protein using a Millipore UltraFree concentrator. Denaturation, reduction, and alkylation were performed as described below.

Purification of His-tagged PGHS-2 by nickel chromatography. Following labeling with [^{125}I]-TID, solubilized microsomal hPGHS-2 was placed in a 15 ml tube and diluted threefold with Buffer A (10 mM phosphate/100 mM NaCl/20 mM imidazole/pH 7.2). Ni-NTA (Qiagen; 1 ml) was added and the tube was incubated at 4°C with gentle shaking for 1 h. Ni-NTA was collected by centrifugation at 1200 rpm in a Beckman table top centrifuge for 2 min and the resin was washed three times for 5 min in wash buffer (10 mM phosphate/500 mM NaCl/30 mM imidazole/0.1% $C_{10}E_6$/pH 7.2). Photoaffinity-labeled hPGHS-2 was eluted from the Ni-NTA by incubation in elution buffer (10 mM phosphate/200 mM NaCl/200 mM imidazole/pH 7.2) for 1 hour at 4°C. Eluted protein was concentrated using a Millipore Ultra-Free concentrator to a volume of approximately 400 µl. Elution salts were removed by dialysis against 10 mM Tris, pH 8.2 prior to denaturation, reduction, and alkylation.

Denaturation, alkylation, reduction, and proteolysis of oPGHS-1 and hPGHS-2. Prior to denaturation, [^{125}I]-TID labeled protein samples were concentrated to a volume of

25 µl. Samples were denatured by incubation in 6M (final concentration) guanidium hydrochloride at 55°C for three hours. Freshly prepared 1M dithiothreitol was then added to a final concentration of 10 mM. After covering with argon, the sample was incubated at 55°C for 2 h. Reduced disulfide bridges were prevented from reforming by the addition of freshly prepared *N*-isopropyl-iodoacetic acid (Molecular Probes, Inc.) and incubation at room temperature in the dark for 1 h. After the alkylation step, samples were dialyzed against 4 L of either 0.05 M NH_4HCO_3/pH 7.9 (for Glu C digestion) or 0.2 M Tris/pH 8.0 (for Lys C digestion) for 12-15 hours in a Fisher 0.5 ml Slide-A-Lyzer (MWCO = 10,000 Da). Prior to proteolysis, samples were concentrated in a SpeedVac to a volume of approximately 50 ul. Proteolytic enzymes endproteinase Lys C or endoproteinase Glu C were added such that the final ratio of protease to PGHS-1/-2 was approximately 1:50. Digestions were performed at 37°C for 20-24 h, with additional endoproteinase Glu C being added every 5 h in the case of hPGHS-2. Proteolytic fragments were analyzed by SDS-PAGE as described below.

SDS-PAGE and analysis of proteolytic products. The following precautions were employed to prevent the N-terminal blockage of peptides separated by SDS-PAGE: (a) highly pure and fresh acrylamide reagents and buffers were used to prepare 16% slab gels, (b) the gels were allowed to polymerize overnight at room temperature before use, and (c) recrystallized SDS was used in all buffers and gels. Proteolytic peptides or untreated controls were separated on the slab gels and transferred to PVDF membranes for Western analysis or autoradiography. In some experiments, portions of gels were silver-stained to observe total protein staining using the BioRad Silver Stain kit. Autoradiography was performed by (a) sandwiching the PVDF membrane between two intensifying screens and

exposing to Amersham Hyperfilm-MP for 4 days at -80°C or (b) exposing the PVDF membrane to a phosphor screen (Molecular Dynamics, Inc.) for two days. In the former case, Stratagene Glogos II fluorescent markers were used to orient the film to the PVDF membrane prior to excision of [^{125}I]-TID labeled peptides. Immunoblot visualization of PGHS-1 or -2 and their proteolytic products was done as described previously. Antibodies against hPGHS-2 were directed against a C-terminal 18-amino acid cassette or amino acids 20-40 at the N-terminus (Santa Cruz Biotechnology, Inc.). An antibody recognizing the N terminus of oPGHS-1 was directed against amino acids 25-35.

Secondary digestion of the PGHS-1 Lys C fragment with Glu C. Using the autoradiograph as a template, the [^{125}I]-TID-labeled oPGHS-1 Lys C fragments were excised from the PVDF membrane using a new, clean razor blade. The excised band was cut into 1 mm^2 squares and placed in a 1.5 ml screw top microcentrifuge tube that had been prewashed once with methanol and twice with water. Glu C digestion buffer (50 µl of 100 mM Tris-Cl/1% reduced TritonX-100/pH 8.0) was added to the squares. The tube was vortexed for 20 s and allowed to incubate at room temperature for 30 min. Endoproteinase Glu C was added to an approximate protease:protein ratio of 1:25. The digestion was performed at 37°C for 24 hours. To recover the proteolytic peptides from the PVDF membrane, sample tubes were vortexed for 10 seconds and placed in a water bath sonicator for 5 min. The tubes were centrifuged for 2 min at 4000 rpm in a Beckman microcentrifuge. The resulting supernatant was transferred to a new, clean microcentrifuge tube. An additional 50 µl aliquot of digestion buffer was added to the squares and subjected to vortexing, sonication, centrifugation, and the supernatant was removed to the new tube. This

was repeated a third time. The sample containing the eluted peptides was concentrated in a SpeedVac to a volume of approximately 50 μl prior to analysis by SDS-PAGE.

Microsequencing of radiolabeled proteolytic peptides. Using the autoradiograph as a template, [^{125}I]-TID labeled peptides were excised from PVDF membranes and cut into 1mm^2 squares. Amino terminal amino acid sequence analysis was performed in collaboration with Dr. Joe Leykam of the Michigan State University Macromolecular Structure Facility using automated Edman degradation in an Applied Biosystem 477A gas phase amino acid sequencer. Phenylthiohydantoin amino acid derivatives were identified by HPLC.

Results

Photolabeling of the PGHS-1 membrane binding domain. Ovine seminal vesicle microsomes containing oPGHS-1 were prepared, preincubated with 80 mM flurbiprofen, and labeled with the hydrophobic, photolabile reagent [^{125}I]-TID. Flurbiprofen prevents the labeling of the oPGHS-1 active site by [^{125}I]-TID [135]. As expected, oPGHS-1 was the major membrane protein in this tissue (Fig. 16). Immunoprecipitation of oPGHS-1 followed by treatment with endoproteinase LysC resulted in an N-terminal fragment with an observed molecular mass of 20 kDa (Fig. 17). This band had been previously observed by Otto and Smith [135] and represents a glycosylated form of the predicted, 16 kDa Lys C product (Fig. 15). The 20 kDa band contains amino acids 25-166 and harbors the predicted membrane binding domain. Similar photolabeling patterns were observed in the presence and absence of glutathione, demonstrating that [^{125}I]-TID labeled only those regions of oPGHS-1 located within the lipid bilayer. Several attempts were made to further digest the 20 kDa band in order to generate a minimum fragment containing the putative membrane binding domain; however, we were never able to obtain enough yield to facilitate sequencing.

The primary structure of oPGHS-1 predicts endoproteinase Glu C products that include a 7.4 kDa peptide containing amino acids 74-140 (Fig. 15). This region includes the predicted membrane binding domain. To determine the regions(s) of oPGHS-1 that contained the [^{125}I]-TID label, photoaffinity-labled PGHS-1 was denatured in guanidium hydrochloride and subjected to exhaustive proteolysis with endoproteinase Glu C. Separation of the Glu C proteolysis products by SDS-PAGE, transfer to PVDF membranes, and subsequent autoradiography revealed a 7.4 kDa, [^{125}I]-TID labeled peptide (Fig. 18).

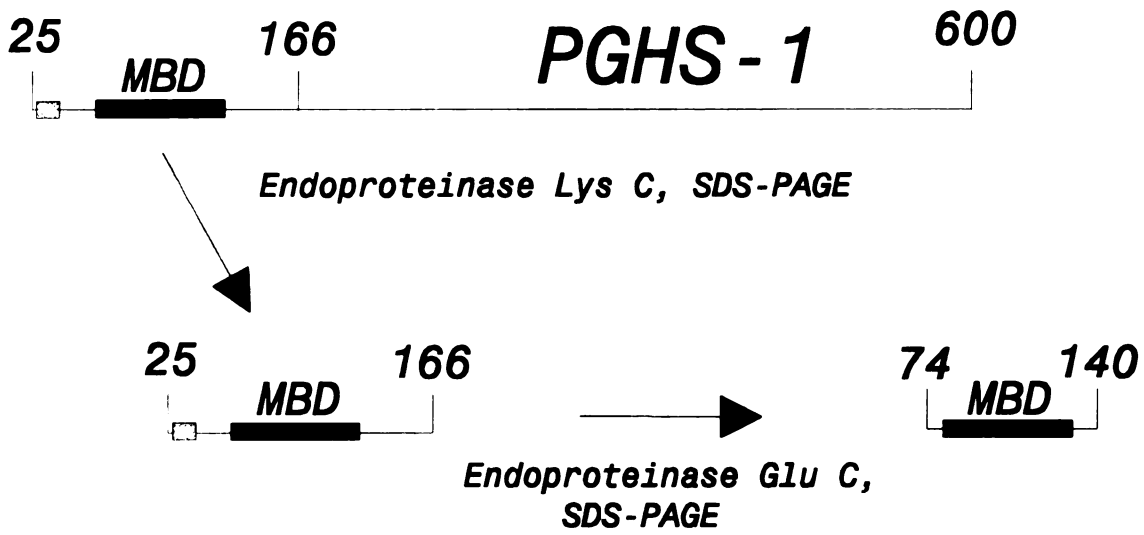

Figure 15. Predicted proteolysis products of oPGHS-1 with Lys C and Glu C. The predicted membrane binding domain is symbolized by a black rectangle and abbreviated MBD. The grey rectangle represents the binding site for the peptide directed antibody against residues 25-35 of oPGHS-1.

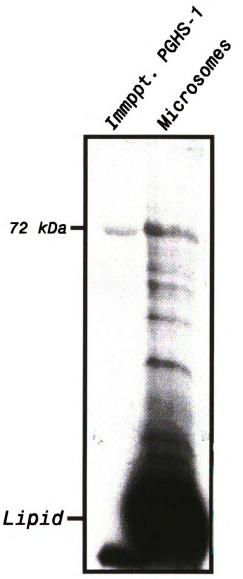

Figure 16. [^{125}I]-TID labeling of ovine seminal vesicle membrane proteins. Seminal vesicle microsomes were labeled with [^{125}I]-TID. One sample was subjected to immunoprecipitation with a monoclonal antibody against PGHS-1. Proteins were separated by SDS polyacrylamide gel electrophoresis, transferred to PVDF membranes, and [^{125}I]-TID labeling was detected by autoradiography.

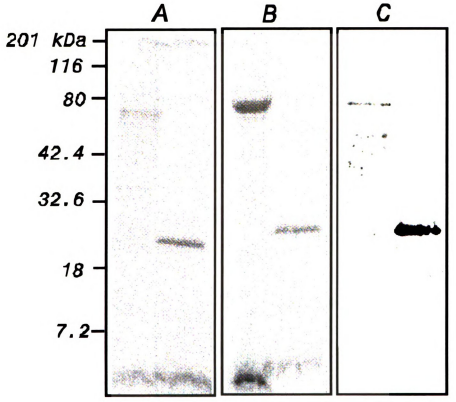

Figure 17. [^{125}I]-TID labeling of an N-terminal peptide of oPGHS-1. After photolabeling ovine seminal vesicles with [^{125}I]-TID, oPGHS-1 was immunoprecipitated from solubilized membrane proteins. Samples were incubated in the presence (right lane) or absence (left lane) of endoproteinase Lys C for 20 h at 37°C. Proteolytic peptides were separated by SDS polyacrylamide gel electrophoresis, transferred to PVDF membranes, and exposed to a phosphor screen to detect [^{125}I]-TID-labeled peptides. *A* and *B*, Autoradiographs of untreated and Lys C treated oPGHS-1 labeled with [^{125}I]-TID in the presence and absence of glutathione, respectively. *C*, Untreated and Lys C treated oPGHS-1 visualized by immunoblot using an antibody against amino acids 25-35 [42].

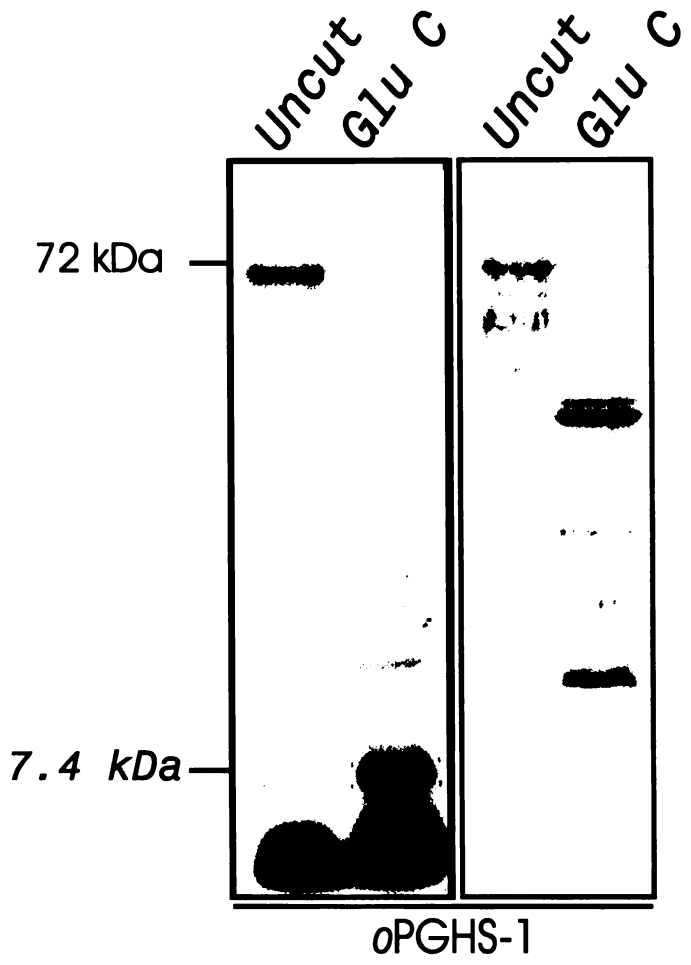

Figure 18. An 7.4 kDa peptide of oPGHS-1 containing the predicted membrane binding domain is labeled by [^{125}I]-TID. [^{125}I]-TID labeled oPGHS-1 samples were incubated in the presence or absence of endoproteinase Glu C for 20 h at 37 °C. Proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes. *Right panel,* Immunoblot of separated proteins with an antibody directed against amino acids 20-40 of hPGHS-2. Note: endoproteinase Glu C reacts nonspecifically with the secondary antibody used in the immunoblot. *Left panel,* Autoradiograph of the same PVDF membrane used for the immunoblot.

```
  10        20          30          40          50
MSRQSISLRF PLLLLLLSPS PVFSADPGAP APVNPCCYYP CQHQGICVRF

         60          70          80          90          100
GLDRYQCDCT RTGYSGPNCT IPEIWTWLRT TLRPSPSFIH FMLTHGRWLW

        110         120         130         140         150
DFVNATFIRD TLMRLVLTVR SNLIPSPPTY NIAHDYISWE SFSNVSYYTR

        160         170         180         190         200
ILPSVPRDCP TPMGTKGKKQ LPDAEFLSRR FLLRRKFIPD PQGTNLMFAF

        210         220         230         240         250
FAQHFTHQFF KTSGKMGPGF TKALGHGVDL GHIYGDNLER QYQLRLFKDG

        260         270         280         290         300
KLKYQMLNGE VYPPSVEEAP VLMHYPRGIP PQSQMAVGQE VFGLLPGLML

        310         320         330         340         350
YATIWLREHN RVCDLLKAEH PTWGDEQLFQ TARLILIGET IKIVIEEYVQ

        360         370         380         390         400
QLSGYFLQLK FDPELLFGAQ FQYRNRIAME FNQLYHWHPL MPDSFRVGPQ

        410         420         430         440         450
DYSYEQFLFN TSMLVDYGVE ALVDAFSRQP AGRIGGGRNI DHHILHVAVD

        460         470         480         490         500
VIKESRVLRL QPFNEYRKRF GMKPYTSFQE LTGEKEMAAE LEELYGDIDA

        510         520         530         540         550
LEFYPGLLLE KCHPNSIFGE SMIEMGAPFS LKGLLGNPIC SPEYWKASTF

        560         570         580         590         600
GGEVGFNLVK TATLKKLVCL NTKTCPYVSF HVPDPRQEDR PGVERPPTEL
```

Figure 19. Primary structure of oPGHS-1. The predicted membrane binding domain predicted by Picot, et al. (34), residues 74-117, is depicted in red typeface. The region of PGHS-1 that corresponds to the 7.4 kDa, [^{125}I]-TID labeled fragment from the autoradiograph in Fig. 17 is underlined. Italicized residues indicate the region of the 7.4 kDa peptide that was confirmed by Edman degradation.

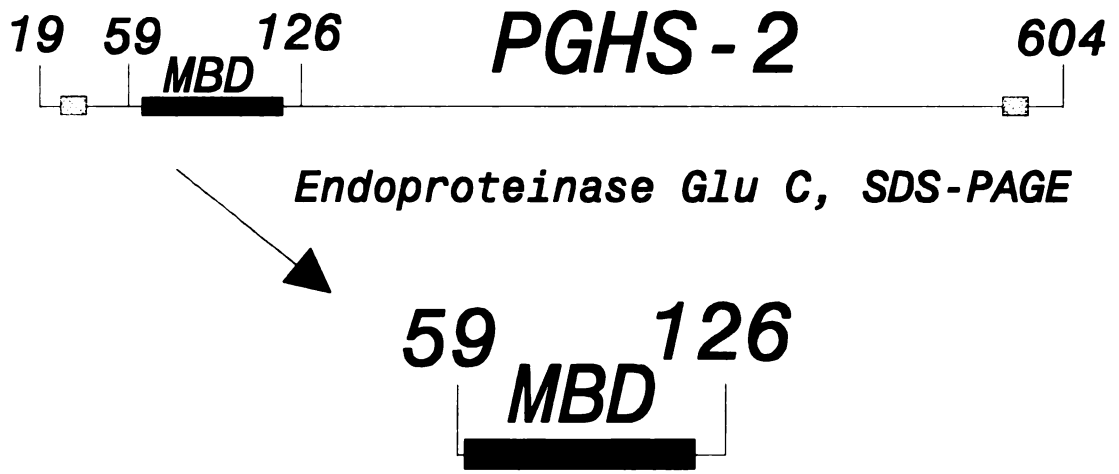

Figure 20. Predicted major proteolysis product of hPGHS-2 with Glu C. The predicted membrane binding domain is symbolized by a black rectangle and abbreviated MBD. The grey rectangle represents the binding site for the anti-peptide antibody directed against amino acids 20-40 of hPGHS-2.

This observation suggested that amino acids 74-140 of oPGHS-1 are associated with the ER in seminal vesicle gland microsomes. Small amounts of incompletely digested PGHS-1 would be predicted to contain the N-terminus of the protein and were recognized by an antibody directed against amino acids 25-35 (Fig. 18).

Peptide mapping of the membrane binding domain of hPGHS-2 with [^{125}I]-TID. hPGHS-2 was engineered to contain a six-histidine tag and expressed in Sf21 insect cells using a baculovirus expression system. The K_m, V_{max}, and subcellular localization of His-tagged PGHS-2 are indistinguishable from those of the native enzyme[1]. To determine which regions of PGHS-2 were associated with the endoplasmic reticulum, microsomal membranes from Sf21 cells were prepared and photoaffinity labeled with [^{125}I]-TID. [^{125}I]-TID-labeled hPGHS-2 was then solubilized and purified by nickel affinity chromatography. Analysis of the purified protein by SDS-PAGE revealed the expected [^{125}I]-TID labeled doublet at 72 and 74 kDa that was recognized by an antibody directed against the N-terminus of PGHS-2 (data not shown).

The primary structure of PGHS-2 predicts endoproteinase Glu C proteolytic products that include an 8 kDa fragment containing amino acids 59-126 (Fig. 20). This region includes the predicted membrane binding domain. To determine the region(s) of PGHS-2 that contained the [^{125}I]-TID label, photoaffinity-labeled hPGHS-2 was denatured in guanidium hydrochloride and subjected to exhaustive proteolysis with endoproteinase Glu C. Separation of the Glu C proteolysis products by SDS-PAGE and subsequent autoradiography revealed an 8 kDa fragment (Fig. 21). This observation suggests that

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[1]T.Smith, D. DeWitt, unpublished results.

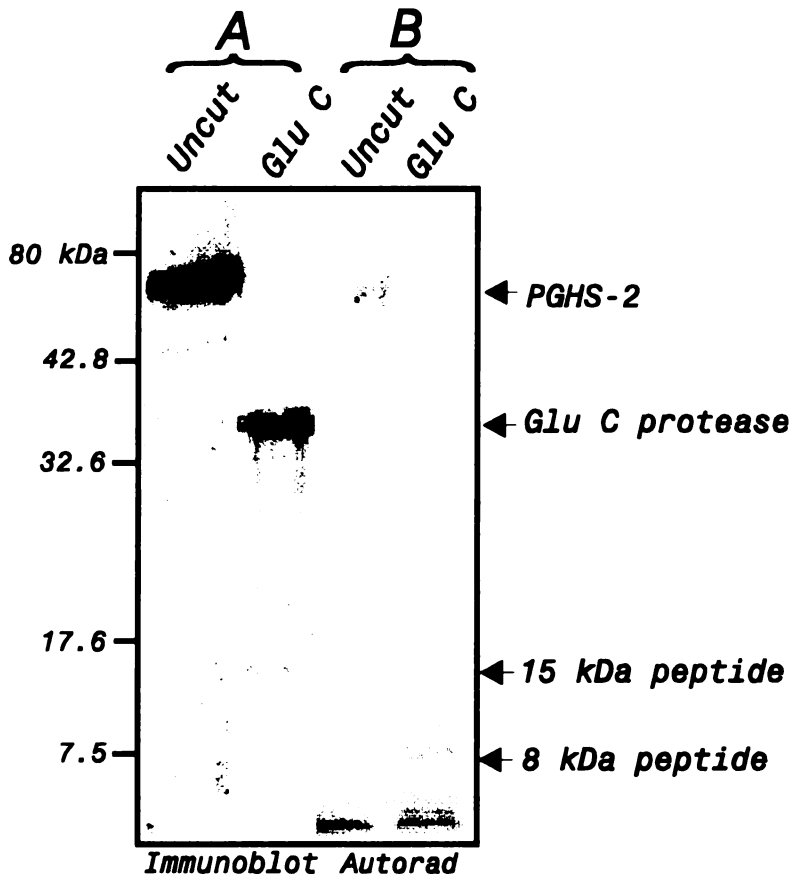

Figure 21. An 8 kDa peptide of hPGHS-2 containing the predicted membrane binding domain is labeled by [^{125}I]-TID. [^{125}I]-TID labeled hPGHS-2 samples were incubated in the presence or absence of endoproteinase Glu C for 20 h at 37°C. Proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes. *A*, Immunoblot of separated proteins with an antibody directed against amino acids 20-40 of hPGHS-2. Note: endoproteinase Glu C reacts nonspecifically with the antibodies used in the immunoblot. *B*, Autoradiograph of the same PVDF membrane used for the immunoblot.

amino acids 59-126 of PGHS-2 are associated with the ER membrane in microsomal preparations. The 8 kDa fragment was not recognized by an antibody directed against amino acids 20-40 of hPGHS-2. Incompletely digested hPGHS-2 peptides (e.g., at 15 kDa) would be predicted to contain the N-terminus of the proteins and were recognized by this antibody (Fig. 21).

To confirm the identity of the 8 kDa photoaffinity labeled fragment from Fig. 21, the radioactive peptides were excised from the PVDF membrane and subjected to 15 rounds of Edman degradation to determine their N-terminal sequence. The N-terminal sequence of this peptide was FLTRIKLFLKPTPNT, which corresponds to amino acids 59-73 of hPGHS-2. As shown in Fig. 22, the 8 kDa band corresponds to amino acids 59-126 in PGHS-2. Thus, amino acids 59-126 of PGHS-2 contain the membrane binding domain. This domain of PGHS-2 corresponds to the four amphipathic helices of PGHS-1 that was predicted by Picot et al. to serve as the membrane association domain [34].

```
        10         20         30         40         50         60
MLARALLLCA VLALSHTANP CCSHPCQNRG VCMSVGFDQY KCDCTRTGFY GENCSTPEFL
        70         80         90        100        110        120
TRIKLFLKPT PNTVHYILTH FKGFWNVVNN IPFLRNAIMS YVLTSRSHLI DSPPTYNADY
       130        140        150        160        170        180
GYKSWEAFSN LSYYTRALPP VPDDCPTPLG VKGKKQLPDS NEIVEKLLLR RKFIPDPQGS
       190        200        210        220        230        240
NMMFAFFAQH FTHQFFKTDH KRGPAFTNGL GHGVDLNHIY GETLARQRKL RLFKDGKMKY
       250        260        270        280        290        300
QIIDGEMYPP TVKDTQAEMI YPPQVPEHLR FAVGQEVFGL VPGLMMYATI WLREHNRVCD
       310        320        330        340        350        360
VLKQEHPEWG DEQLFQTSRL ILIGETIKIV IEDYVQHLSG YHFKLKFDPE LLFNKQFQYQ
       370        380        390        400        410        420
NRIAAEFNTL YHWHPLLPDT FQIHDQKYNY QQFIYNNSIL LEHGITQFVE SFTRQIAGRV
       430        440        450        460        470        480
AGGRNVPPAV QKVSQASTDQ SRQMKYQSFN EYRKRFMLKP YESFEELTGE KEMSAELEAL
       490        500        510        520        530        540
YGDIDAVELY PALLVEKPRP DAIFGETMVE VGAPFSLKGL MGNVICSPAY WKPSTFGGEV
       550        560        570        580        590        600
GFQIINTASI QSLICNNVKG CPFTSFSVPD PELIKTVTIN ASSSRSGLDD INPTVLLKER

STEL
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Figure 22. Primary structure of hPGHS-2. The predicted membrane binding domain predicted by Picot, et al. (34), residues 58-108, is depicted in red typeface. The region of PGHS-2 that corresponds to the 8 kDa, [^{125}I]-TID labeled fragment from the autoradiograph in Fig. 19 is underlined. Italicized residues indicate the region of the 8 kDa peptide that was confirmed by Edman degradation.

Discussion

PGHS-1 and PGHS-2 are integral membranes of the endoplasmic reticulum and nuclear envelope. The major aim of this study was to determine which regions of these proteins interact with the membrane bilayer. Determination of the membrane associated regions of PGHS-1 and -2 is of interest because the enzymes do not have classical transmembrane domains. Instead, these proteins were hypothesized by Picot, et al. to associate with the ER through four amphipathic alpha helices [34]. We have used the hydrophobic, photoactivatable compound [^{125}I]-TID to show that the membrane binding domains of oPGHS-1 and hPGHS-2 reside within amino acids 74-140 and 59-126, respectively. The present study extends previous work from our laboratory demonstrating that the membrane binding domain of oPGHS-1 is between amino acids 25-166. In addition, these studies provide the first biochemical identification of the membrane association domain of hPGHS-2. Our results indicate that both isozymes interact with the ER through the four-helix region predicted by X-ray crystallographic analysis of ovine PGHS-1 (Fig 6).

Recent crystallographic analysis of a bacterial squalene cyclase has suggested that membrane proteins other than PGHS-1 and -2 may interact with cellular membranes in a similar fashion [40]. The overall protein folds of PGHS-1 and bacterial squalene cyclase are quite different. However, the overall shapes of the two proteins are similar. Both proteins are homodimeric and possess hydrophobic patches that protrude away from the hydrophilic catalytic domains.

It is interesting that the only two known protein structures with apparent monotopic membrane domains are lipid biosynthetic enzymes. Monotopic membrane binding domains

like those of the PGHSs and squalene cyclase appear to be positioned in such a way to allow direct substrate access to the active site. This would be thermodynamically favorable since hydrophobic substrates like arachidonate and squalene are not likely to be released directly into an aqueous environment. Thus, the membrane binding domains of PGHS and squalene cyclase appear to function as (a) membrane anchors, and (b) continuous hydrophobic tunnels from the site of substrate release to the active site. It is conceivable that other enzymes whose substrate comes directly from the lipid bilayer may use a similar membrane association strategy.

We have shown previously that PGHS-1 and -2 are both present in the ER and the inner and outer membranes of the nuclear envelope in similar proportions [68]. This work suggested that the enzymes are not physically separated by compartmentalization in different cellular membranes, but did not rule out the possibility that PGHS-1 and PGHS-2 are associated with different phospholipid microdomains within the ER and nuclear envelope. The catalytic domains of PGHS-1 and -2 share 70% of their primary structure, while the membrane binding domains of the enzymes are only 38% identical. Several non-conservative amino acid substitutions exist in PGHS-2 at positions that may interact with polar phospholipid heads of the membrane bilayer. An interesting question is whether the sequence differences in the membrane binding domains of PGHS-1 and -2 may promote association of each isozyme with different phospholipid microdomains within cellular membranes.

Interactions between membrane proteins and the polar head groups of phospholipids have been shown to be of biological importance [159-161]. In addition, the induction of phospholipid microdomains upon exposure of biological membranes to physiological

concentrations of calcium imply that phospholipid microdomains may form *in vivo* [162]. The phospholipid microenvironment immediately surrounding an integral membrane protein may affect its biological activity by promoting or restricting access of the protein to various components of the cellular membrane. In the case of PGHS-1 and -2, the significant sequence differences at the protein-phospholipid interface may confer differential interactions with phospholipid microdomains with which the proteins associate *in vivo*.

We conclude that PGHS-1 and -2 interact with the ER through a novel domain consisting of four amphipathic alpha helices. This region was previously hypothesized by Picot, et al. to serve as the membrane binding domain.

APPENDIX

APPENDIX A

Future Directions

One interesting question arising from the work presented in Chapter II concerns the topological properties and trafficking of integral membrane proteins in the nuclear envelope. Our immunogold localization experiments led us to speculate that the lumenal orientation of PGHS-1 and -2 allows them to reach the inner nuclear membrane by lateral diffusion. We plan to use immunoelectron microscopy to examine other ER membrane proteins in the nuclear envelope. Of particular interest will be the distribution of cytoplasmically-oriented ER proteins like the sterol regulatory element binding proteins (SREBPs) and thromboxane A_2 synthase (TxS). One may predict that these proteins, which have large globular domains on the cytoplasmic surface of the ER, are prevented from reaching the inner nuclear membrane due to the physical barrier of the nuclear pore complex (Fig. 13). Determining the nuclear envelope distributions of SREBPs, TxS, and other proteins with similar membrane topography will provide important information on two fronts. First, we will have an improved understanding of the cell biology of integral membrane proteins and the topological properties of proteins needed to navigate the different membranes of the nuclear envelope. Secondly, these studies may provide some insight into the possibile existence of a nuclear prostaglandin signaling system. We have shown that PGHS-1 and -2 are present on the inner nuclear membrane; however, their product, PGH_2, must be converted to active prostaglandins by downstream synthases like TxS and PGI_2-synthase. The distribution of

these prostaglandin synthases in the nuclear envelope is currently unknown but are likely to play an important role in eicosanoid biosynthesis that may take place at the nuclear envelope.

Much of the speculation surrounding the separation of the PGHS-1 and -2 activities has focused on the cell biological aspects of the enzymes. Differential localization, coupling to specific PLA_2s, FLAP-like arachidonate shuttling proteins, and localization to different phospholipid microdomains have all been discussed as possible ways to separate the activities of the PGHSs. However, it is possible that cell biological properties have little to do with separating the activities of PGHS-1 and -2 in living cells. There is some evidence that biochemical properties of the two enzymes allow them to be exclusively active under different conditions. Specifically, substrate-dependent allosteric effects have been shown to produce a 2-4-fold greater rate of prostaglandin production by PGHS-2 compared to PGHS-1 at arachidonate concentrations below 0.5 μM (143). In effect, PGHS-2 could be preferentially activated by a stimulation that released arachidonate at levels below the activity threshold of PGHS-1. This would account for the ability of the enzymes to be exclusively active despite the seemingly ubiquitous release of substrate.

The work hypothesizing different substrate thresholds for PGHS-1 and -2 was performed using purifed proteins. It may be possible to examine the substrate thresholds required for activity in living cells. A fluorescent histochemical assay has been used by our laboratory to visualize the synthesis of PGH_2 by PGHS-1 and -2. This assay works by adding a fluorescent peroxidase co-substrate that serves as a reducing substrate for the peroxidase activity of the PGHSs. One is therefore able to visualize the activities of PGHS-1 and -2 together and individually through the use of isozyme-specific inhibitors. This histochemical

technique would allow one to determine which PGHS enzyme was active under various stimulation conditions and at different concentrations of exogenously-added arachidonic acid. Therefore, these studies would enable one to test the "substrate threshold" hypothesis in a living experimental system.

As discussed above, the release of arachidonic acid and its subsequent availability to PGHS-1 and -2 is a complex process. The sheer number of mammalian PLA_2s (over ten) makes sorting out their individual physiological roles very difficult. However, the relationships between the various PLA_2s and the PGHS isozymes are clearly important to eicosanoid signaling. It may be useful to develop an experimental system that would permit the use of genetics to analyze the roles of proteins involved in eicosanoid biosynthesis. Genetic analysis allows one to dissect complex signaling pathways by screening for organisms deficient in a particular portion of the pathway. Furthermore, crosstalk between a pathway of interest and other signaling pathways can be detected by genetic crossing of two or more mutant strains. Most experimental systems of genetic analysis employ the use of model organisms.

The nematode *Caenorhabditis elegans* may present a model system that would be useful to genetically analyze prostanoid-like signaling pathways. This organism is a 959-cell roundworm that has been used for over 20 years to study many aspects of developmental biology. Its complete genomic sequence will be available in late 1998. *C. elegans* contain both arachidonic (20:4) and eicosapentanoic acid (20:5), which are precursors for eicosanoids in mammalian systems (168). They also contain DNA sequences that show sequence homology to phospholipases A_2. If one could show that a prostanoid-like signaling pathway existed in *C. elegans*, then its genetic tractability might prove to be useful in disecting the

complex pathways controlled by lipid mediators. The existence of such a pathway in *C. elegans* has neither been demonstrated nor looked for. However, the infastructure is present in this organsism and the initial studies to determine the presence of lipid oxygenases are straightforward biochemical assays using radiolabeled substrates and product analysis. If successful, this sytem could provide insight into prostanoid biology as well as the role of lipid mediators in development of metazoan organisms.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Smith, W.L., R.M. Garavito, and D.L. DeWitt, Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2 Prostaglandin endoperoxide H synthases-1 and -2. *J. Biol.Chem.*, 1996. **271**(52): p. 33157-60.

2. Smith, W.L. and L.J. Marnett, Prostaglandin endoperoxide synthase: structure and catalysis. *Biochim. Biophys. Acta*, 1991. **1083**(1): p. 1-17.

3. Hemler, M. and W.E. Lands, Purification of the cyclooxygenase that forms prostaglandins. Demonstration of two forms of iron in the holoenzyme. *J. Biol. Chem.*, 1976. **251**(18): p. 5575-9.

4. Herschman, H.R., Prostaglandin synthase 2. *Biochim. Biophys. Acta,* 1996. **1299**(1): p. 125-40.

5. Burr, G. and Burr, M.M., A new deficiency disease produced by the rigid exclusion of fat from the diet. *J. Biol. Chem.*, 1929. **82**(1): p. 345-367.

6. Burr, G. and Burr., M.M, On the Nature and role of the fatty acids essential in nutrition. *J. Biol. Chem.*, 1930. **86**(1): p. 587-619.

7. Morham, S.G., *et al.*, Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell*, 1995. **83**(3): p. 473-82.

8. Langenbach, R., *et al.*, Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell*, 1995. **83**(3): p. 483-92.

9. Dunham, E.W., Balasingham, M., Privett, O.S., and Nickell, E.C., Effects of essential fatty acid deficiency on prostaglandin synthesis and fatty acid composition in renal medulla. *Lipids*, 1978. **13**: p. 892-897.

10. von Euler, U., Prostaglandins: Historical remarks, in *Prog. Lipid Res.* 1982. p. xxxi-xxxv.

11. von Euler, U.S., On the specific vasodilating and plain muscle stimulating substances from accessory genital glands in man and certain animals. *J. Physiol.*, 1936. **88**: p. 213-234.

12. Kurzrok, R.a.L., C.C., Biochemical studies on human semen. The action of semen on the human uterus. *Proc. Soc. Exp. Biol. Med.*, 1930. **28**: p. 268-272.

13. Willis, A.L., Prosaglandins and Related Lipids. 1 ed. CRC Handbook of Eicosanoids.

Vol. I. 1987, Boca Raton: CRC Press, Inc. 314.

14. Sinclair, H.M., Essential Fatty Acids. 1 ed. 1958, London: Butterworths Scientific Publications. 264.

15. Bergstrom, S., Rhyhage, R., Samuelsson, B., and Sjovall, J., The structures of prostaglandins E_1, $F_{1\alpha}$ and $F_{1\beta}$. *J. Biol. Chem.*, 1963. **238**: p. 3555-3564.

16. Bergstrom, S., Danielsson, H., and Samuelsson, B., The enzymatic formation of prostaglandin E_2 from arachidonic acid. *Biochimica Biophysica Acta*, 1964. **90**: p. 207-210.

17. Van Dorp, D.A., Beerthuis, R.K., Nugteren, D.H., and Vonkeman, H., The biosynthesis of prostaglandins. *Biochimica Biophysica Acta*, 1964. **90**: p. 204-297.

18. Van der Ouderaa, F.J., *et al.*, Purification and characterisation of prostaglandin endoperoxide synthetase from sheep vesicular glands. *Biochim Biophys Acta*, 1977. **487**(2): p. 315-31.

19. Miyamoto, T., *et al.*, Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J. Biol. Chem.*, 1976. **251**(9): p. 2629-36.

20. Vane, J.R., Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat. New Biol.*, 1971. **231**(25): p. 232-5.

21. Ferreira, S.H., S. Moncada, and J.R. Vane, Indomethacin and aspirin abolish prostaglandin release from the spleen. *Nat. New Biol.*, 1971. **231**(25): p. 237-9.

22. DeWitt, D.L. and W.L. Smith, Cloning of sheep and mouse prostaglandin endoperoxide synthases. *Methods Enzymol.*, 1990. **187**: p. 469-79.

23. Smith, W.L., *et al.*, Structure-function relationships in sheep, mouse, and human prostaglandin endoperoxide G/H synthases. *Adv. Prostaglandin Thromboxane Leukot. Res.*, 1990. **20**: p. 14-21.

24. Hla, T. and K. Neilson, Human cyclooxygenase-2 cDNA. *Proc. Natl. Acad. Sci. U S A*, 1992. **89**(16): p. 7384-8.

25. Shimokawa, T. and W.L. Smith, Prostaglandin endoperoxide synthase. The aspirin acetylation region. *J. Biol. Chem.*, 1992. **267**(17): p. 12387-92.

26. Laneuville, O., *et al.*, Fatty acid substrate specificities of human prostaglandin-endoperoxide H synthase-1 and -2. Formation of 12-hydroxy-(9Z, 13E/Z, 15Z)-octadecatrienoic acids from alpha-linolenic acid. J. Biol.Chem., 1995. **270**(33): p.

19330-6.

27. DeWitt, D.L., *et al.*, The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. *J. Biol. Chem.*, 1990. **265**(9): p. 5192-8.

28. Rollins, T.E. and W.L. Smith, Subcellular localization of prostaglandin-forming cyclooxygenase in Swiss mouse 3T3 fibroblasts by electron microscopic immunocytochemistry. *J. Biol. Chem*, 1980. **255**(10): p. 4872-5.

29. Kennedy, I., *et al.*, Studies on the characterization of prostanoid receptors. *Adv. Prostaglandin Thromboxane Leukot. Res.*, 1983. **11**: p. 327-32.

30. Coleman, R.A., *et al.*, Characterisation of the prostanoid receptors mediating contraction of guinea-pig isolated trachea: Studies on the characterization of prostanoid receptors. *Prostaglandins,* 1985. **29**(3): p. 363-75.

31. Fu, J.Y., *et al.*, The induction and suppression of prostaglandin H2 synthase (cyclooxygenase) in human monocytes. *J. Biol. Chem.*, 1990. **265**(28): p. 16737-40.

32. Xie, W.L., *et al.*, Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci. U S A*, 1991. **88**(7): p. 2692-6.

33. Kujubu, D.A., *et al.*, TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J. Biol. Chem.,* 1991. **266**(20): p. 12866-72.

34. Picot, D., P.J. Loll, and R.M. Garavito, The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1. *Nature*, 1994. **367**(6460): p. 243-9.

35. Kurumbail, R.G., *et al.*, Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature,* 1996. **384**(6610): p. 644-8.

36. Meade, E.A., W.L. Smith, and D.L. DeWitt, Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. *J. Biol. Chem.*, 1993. **268**(9): p. 6610-4.

37. Laneuville, O., *et al.*, Differential inhibition of human prostaglandin endoperoxide H synthases-1 and -2 by nonsteroidal anti-inflammatory drugs. *J. Pharmacol. Exp. Ther.*, 1994. **271**(2): p. 927-34.

38. DeWitt, D.L., *et al.*, PGH synthase isoenzyme selectivity: the potential for safer nonsteroidal antiinflammatory drugs. *Am. J. Med.*, 1993. **95**(2a): p. 40s-44s.

39. Roth, G.J., C.J. Siok, and J. Ozols, Structural characteristics of prostaglandin synthetase from sheep vesicular gland. *J. Biol. Chem.*, 1980. **255**(4): p. 1301-4.

40. Wendt, K.U., K. Poralla, and G.E. Schulz, Structure and function of a squalene cyclase. *Science*, 1997. **277**(5333): p. 1811-5.

41. Otto, J.C., D.L. DeWitt, and W.L. Smith, N-glycosylation of prostaglandin endoperoxide synthases-1 and -2 and their orientations in the endoplasmic reticulum. *J. Biol. Chem,*, 1993. **268**(24): p. 18234-42.

42. Otto, J.C. and W.L. Smith, The orientation of prostaglandin endoperoxide synthases-1 and -2 in the endoplasmic reticulum. *J. Biol. Chem.*, 1994. **269**(31): p.19868-75.

43. Smith, W.L. and D.L. Dewitt, Prostaglandin endoperoxide H synthases-1 and -2. *Adv. Immunol.*, 1996. **62**: p. 167-215.

44. Silvia, W.J., Brockman, J.A., Kaminski, M.A., DeWitt, D.L., and Smith, W.L., Prostaglandin endoperoxide synthase in seminal vesicles. *Mol. Androl.*, 1994. **6**: p. 197-207.

45. Brannon, T.S., *et al.*, Prostacyclin synthesis in ovine pulmonary artery is developmentally regulated by changes in cyclooxygenase-1 gene expression. *J. Clin. Invest.*, 1994. **93**(5): p. 2230-5.

46. Xu, X.M., *et al.*, Involvement of two Sp1 elements in basal endothelial prostaglandin H synthase-1 promoter activity. *J. Biol. Chem.*, 1997. **272**(11): p. 6943-50.

47. Xu, X.M., *et al.*, Transcriptional regulation of endothelial constitutive PGHS-1 expression by phorbol ester. *Am. J. Physiol.*, 1996. **270**(1 Pt 1): p. C259-64.

48. Breder, C.D., DeWitt, D.L., Kraig, R.P., Characterization of inducible cyclooxygenase in rat brain. *Journal of Comparative Neurology*, 1995. **355**: p. 296-315.

49. DeWitt, D.L. and E.A. Meade, Serum and glucocorticoid regulation of gene transcription and expression of the prostaglandin H synthase-1 and prostaglandin H synthase-2 isozymes. *Arch. Biochem. Biophys.*, 1993. **306**(1): p. 94-102.

50. Murakami, M., *et al.*, IgE-dependent activation of cytokine-primed mouse cultured mast cells induces a delayed phase of prostaglandin D2 generation via prostaglandin endoperoxide synthase-2. *J. Immunol.*, 1995. **155**(9): p. 4445-53.

51. Xie, W., Fletcher, B.S., Andersen, R.D., and Herschman, H.R., v-src induction of the TIS10/PGS2 prostaglandin synthase gene is mediated by an ATF/CRE transcription

response element. *Mol. Cell Biol.*, 1994. **14**: p. 6531-6539.

52. Inoue, H., Nanayama, T., Hara, S., Yokoyama, C., and Tanabe, T., The cyclic AMP response element plays an essential role in the expression of the human prostaglandin-endoperoxide synthase 2 gene in differentiated U937 monocytic cells. *FEBS Lett.*, 1994. **350**: p. 51-54.

53. Masferrer, J.L., *et al.*, In vivo glucocorticoids regulate cyclooxygenase-2 but not cyclooxygenase-1 in peritoneal macrophages. *J. Pharmacol. Exp. Ther.*, 1994. **270**(3): p. 1340-4.

54. Marnett, L.J., Aspirin and the potential role of prostaglandins in colon cancer. *Cancer Res.*, 1992. **52**(20): p. 5575-89.

55. Tsujii, M. and R.N. DuBois, Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell*, 1995. **83**(3): p. 493-501.

56. Kinzler, K.W. and B. Vogelstein, Lessons from hereditary colorectal cancer. *Cell*, 1996. **87**(2): p. 159-70.

57. Oshima, M., *et al.*, Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell*, 1996. **87**(5): p. 803-9.

58. Giardiello, F.M., *et al.*, Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N. Engl. J. Med.*, 1993. **328**(18): p. 1313-6.

59. Leslie, C.C., Properties and Regulation of Cytosolic Phospholipase A_2. *J. Biol. Chem.*, 1997. **272**: p. 16709-16712.

60. Tischfield, J.A., A reassessment of the low molecular weight phospholipase A_2 gene family in mammals. *J. Biol. Chem.*, 1997. **272**(28): p. 17247-50.

61. Schievella, A.R., *et al.*, Calcium-mediated translocation of cytosolic phospholipase A_2 to the nuclear envelope and endoplasmic reticulum. *J. Biol. Chem.*, 1995. **270**(51): p. 30749-54.

62. Glover, S., *et al.*, Translocation of the 85-kDa phospholipase A_2 from cytosol to the nuclear envelope in rat basophilic leukemia cells stimulated with calcium ionophore or IgE/antigen [published erratum appears in J Biol Chem 1995 Sep 1;270(35):20870]. *J. Biol. Chem.*, 1995. **270**(25): p. 15359-67.

63. Borsch Haubold, A.G., *et al.*, Identification of the phosphorylation sites of cytosolic phospholipase A_2 in agonist-stimulated human platelets and HeLa cells. *J. Biol.*

Chem., 1998. **273**(8): p. 4449-58.

64. Lin, L.L., *et al.*, $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell,* 1993. **72**(2): p. 269-78.

65. Nemenoff, R.A., *et al.*, Phosphorylation and activation of a high molecular weight form of phospholipase A_2 by p42 microtubule-associated protein 2 kinase and protein kinase C. *J. Biol. Chem.*, 1993. **268**(3): p. 1960-4.

66. Sierra Honigmann, M.R., J.R. Bradley, and J.S. Pober, "Cytosolic" phospholipase A_2 is in the nucleus of subconfluent endothelial cells but confined to the cytoplasm of confluent endothelial cells and redistributes to the nuclear envelope and cell junctions upon histamine stimulation. *Lab. Invest.*, 1996. **74**(3): p. 684-95.

67. Morita, I., *et al.*, Different intracellular locations for prostaglandin endoperoxide H synthase-1 and -2. *J. Biol. Chem.*, 1995. **270**(18): p. 10902-8.

68. Spencer, A.G., Woods, J.W., Arakawa, T., Singer, I., and Smith, W.L., Subcellular localization of prostaglandin endoperoxide H synthases-1 and -2 by immunoelectron microscopy. *J. Biol. Chem.*, 1998. **273**: p. 9886-9893.

69. Woods, J.W., *et al.*, 5-lipoxygenase and 5-lipoxygenase-activating protein are localized in the nuclear envelope of activated human leukocytes. *J. Exp. Med.*, 1993. **178**(6): p. 1935-46.

70. Woods, J.W., *et al.*, 5-Lipoxygenase is located in the euchromatin of the nucleus in resting human alveolar macrophages and translocates to the nuclear envelope upon cell activation. *J. Clin. Invest.*, 1995. **95**(5): p. 2035-46.

71. Lin, Y., *et al.*, Docking phospholipase A_2 on membranes using electrostatic potential-modulated spin relaxation magnetic resonance. *Science*, 1998. **279**(5358): p. 1925-9.

72. Kudo, I., *et al.*, Mammalian non-pancreatic phospholipases A_2. *Biochim. Biophys. Acta*, 1993. **1170**(3): p. 217-31.

73. Murakami, M., I. Kudo, and K. Inoue, Secretory phospholipases A_2. J. Lipid Mediat. Cell Signal., 1995. **12**(2-3): p. 119-30.

74. Balsinde J, B.M., Dennis EA, Functional coupling between secretory phospholipase A_2 and cyclooxygenase-2 and its regulation by cytosolic group IV phospholipase A_2. Proc. Natl. Acad. Sci. U S A, 1998. **95**(14): p. 7951-7956.

75. Reddy, S.T. and H.R. Herschman, Ligand-induced prostaglandin synthesis requires expression of the TIS10/PGS-2 prostaglandin synthase gene in murine fibroblasts and

macrophages. J. Biol. Chem., 1994. **269**(22): p. 15473-80.

76. Kirtikara, K., *et al.*, Compensatory prostaglandin E2 biosynthesis in cyclooxygenase 1 or 2 null cells. J. Exp. Med., 1998. **187**(4): p. 517-23.

77. Reddy, S.T. and H.R. Herschman, Transcellular prostaglandin production following mast cell activation is mediated by proximal secretory phospholipase A_2 and distal prostaglandin synthase 1. J. Biol. Chem., 1996. **271**(1): p. 186-91.

78. Reddy, S.T. and H.R. Herschman, Prostaglandin synthase-1 and prostaglandin synthase-2 are coupled to distinct phospholipases for the generation of prostaglandin D2 in activated mast cells. *J. Biol. Chem.*, 1997. **272**(6): p. 3231-7.

79. Bingham, C.O., 3rd, *et al.*, A heparin-sensitive phospholipase A_2 and prostaglandin endoperoxide synthase-2 are functionally linked in the delayed phase of prostaglandin D_2 generation in mouse bone marrow-derived mast cells. J. Biol. Chem., 1996. **271**(42): p. 25936-44.

80. Reddy, S.T., *et al.*, Analysis of the secretory phospholipase A_2 that mediates prostaglandin production in mast cells. *J. Biol. Chem.*, 1997. **272**(21): p. 13591-6.

81. Balsinde, J., *et al.*, Inhibition of calcium-independent phospholipase A_2 prevents arachidonic acid incorporation and phospholipid remodeling in P388D1 macrophages. Proc. Natl. Acad. Sci. U S A, 1995. **92**(18): p. 8527-31.

82. Balboa, M.A., *et al.*, Novel group V phospholipase A_2 involved in arachidonic acid mobilization in murine P388D1 macrophages. *J. Biol. Chem.*, 1997. **271**(50): p. 32381-4.

83. Kuwata, H., Yoshihito Nakatani, Makoto Murakami, and Ichiro Kudo, Cytosolic Phospholipase A_2 Is Required for Cytokine-induced Expression of Type IIA Secretory Phospholipase A_2 That Mediates Optimal Cyclooxygenase-2-dependent Delayed Prostaglandin E2 Generation in Rat 3Y1 Fibroblasts. *J. Biol. Chem.*, 1998. **273**: p. 1733-1740.

84. Murakami, M., Satoko Shimbara, Terumi Kambe, Hiroshi Kuwata, Michelle V. Winstead, Jay A. Tischfield, and Ichiro Kudo, The Functions of Five Distinct Mammalian Phospholipase A_2s in Regulating Arachidonic Acid Release. *J. Biol. Chem.*, 1998. **273**: p. 14411-14423.

85. Dixon, R.A., *et al.*, Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature*, 1990. **343**(6255): p. 282-4.

86. Reid, G.K., *et al.*, Correlation between expression of 5-lipoxygenase-activating protein, 5-lipoxygenase, and cellular leukotriene synthesis. *J. Biol. Chem.*, 1990.

265(32): p. 19818-23.

87. Kanai, N., *et al.*, Identification and characterization of a prostaglandin transporter. *Science*, 1995. **268**(5212): p. 866-9.

88. Lu, R., *et al.*, Cloning, in vitro expression, and tissue distribution of a human prostaglandin transporter cDNA(hPGT). *J. Clin. Invest.*, 1996. **98**(5): p. 1142-9.

89. Ushikubi, F., M. Hirata, and S. Narumiya, Molecular biology of prostanoid receptors; an overview. *J. Lipid Mediat. Cell. Signal.*, 1995. **12**(2-3): p. 343-59.

90. Mais, D.E., T.A. True, and M.J. Martinelli, Characterization by photoaffinity labelling of the human platelet thromboxane A_2/prostaglandin H2 receptor: evidence for N-linked glycosylation. *Eur. J. Pharmacol.*, 1992. **227**(3): p. 267-74.

91. Murray, R., E. Shipp, and G.A. FitzGerald, Prostaglandin endoperoxide/thromboxane A_2 receptor desensitization. Cross-talk with adenylate cyclase in human platelets. *J. Biol. Chem.*, 1990. **265**(35): p. 21670-5.

92. Murray, R. and G.A. FitzGerald, Regulation of thromboxane receptor activation in human platelets. Proc. Natl. Acad. Sci. U S A, 1989. **86**(1): p. 124-8.

93. Habib, A., *et al.*, Rapid, agonist-dependent phosphorylation in vivo of human thromboxane receptor isoforms. Minimal involvement of protein kinase C. *J. Biol. Chem.*, 1997. **272**(11): p. 7191-200.

94. Nishigaki, N., M. Negishi, and A. Ichikawa, Two Gs-coupled prostaglandin E receptor subtypes, EP2 and EP4, differ in desensitization and sensitivity to the metabolic inactivation of the agonist. *Mol. Pharmacol.*, 1996. **50**(4): p. 1031-7.

95. Ichikawa, A., Y. Sugimoto, and M. Negishi, Molecular aspects of the structures and functions of the prostaglandin E receptors. *J. Lipid Mediat. Cell Signal.*, 1996. **14**(1-3): p. 83-7.

96. Watkins, W.D., Peterson, M.B., Fletcher, J.R., Prostaglandins in Clinical Practice. 1989, New York: Raven Press. 263.

97. Watabe, A., Y Sugimoto, A Honda, A Irie, T Namba, M Negishi, S Ito, S Narumiya, and A Ichikawa, Cloning and expression of cDNA for a mouse EP1 subtype of prostaglandin E receptor. *J. Biol. Chem.*, 1993. **268**: p. 20175-20178.

98. Wahli, W. and E. Martinez, Superfamily of steroid nuclear receptors: positive and negative regulators of gene expression. *FASEB J.*, 1991. **5**(9): p. 2243-9.

99. Tontonoz, P., *et al.*, mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. *Genes Dev.*, 1994. **8**(10): p. 1224-34.

100. Lehmann, J.M., *et al.*, An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J. Biol. Chem.*, 1995. **270**(22): p. 12953-6.

101. Spiegelman, B.M., *et al.*, PPAR gamma and the control of adipogenesis. *Biochimie,* 1997. **79**(2-3): p. 111-2.

102. Kliewer, S.A., *et al.*, A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell*, 1995. **83**(5): p. 813-9.

103. Yu, K., *et al.*, Differential activation of peroxisome proliferator-activated receptors by eicosanoids. *J. Biol. Chem.*, 1995. **270**(41): p. 23975-83.

104. Forman, B.M., *et al.*, 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell,* 1995. **83**(5): p. 803-12.

105. Ricote, M., *et al.*, The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature,* 1998. **391**(6662): p. 79-82.

106. Jiang, C., A.T. Ting, and B. Seed, PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature*, 1998. **391**(6662): p. 82-6.

107. Kim, J.B., Wright, H.M., Wright, M, and Spiegelman, B.M., ADD1/SREBP1 activates $PPAR_{\gamma}$ through the production of endogenous ligand. *Proc. Natl. Acad. Sci. USA*, 1998. **95**: p. 4333-4337.

108. Eling, T.E. and A.I. Ally, Pulmonary biosynthesis and metabolism of prostaglandins and related substances. *Environ. Health Perspect.*, 1984. **55**: p. 159-68.

109. Matsuura, S., *et al.*, Immunoelectron microscopy of the outer membrane of rat hepatocyte nuclear envelopes in relation to the rough endoplasmic reticulum. *Cell Struct. Funct.,* 1983. **8**(1): p. 1-9.

110. Martin, L., C. Crimaudo, and L. Gerace, cDna cloning and characterization of lamina-associated polypeptide 1c (Lap1c), an integral protein of the inner nuclear membrane. *J. Biol. Chem.*, 1995. **270**(15): p. 8822-8.

111. Furukawa, K., *et al.*, Cloning of a cDNA for lamina-associated polypeptide 2 (LAP2) and identification of regions that specify targeting to the nuclear envelope. *Embo. J.*, 1995. **14**(8): p. 1626-36.

112. Bione, S., *et al.*, Identification of a novel X-linked gene responsible for Emery-Dreifuss muscular dystrophy. *Nat. Genet.*, 1994. **8**(4): p. 323-7.

113. Nagano, A., *et al.*, Emerin deficiency at the nuclear membrane in patients with Emery-Dreifuss muscular dystrophy. *Nat. Genet.*, 1996. **12**(3): p. 254-9.

114. Soullam, B. and H.J. Worman, Signals and structural features involved in integral membrane protein targeting to the inner nuclear membrane. *J. Cell. Biol.*, 1995. **130**(1): p. 15-27.

115. Smith, S. and G. Blobel, The first membrane spanning region of the lamin B receptor is sufficient for sorting to the inner nuclear membrane. J. Cell. Biol., 1993. **120**(3): p. 631-7.

116. Ellenberg, J., *et al.*, Nuclear membrane dynamics and reassembly in living cells: targeting of an inner nuclear membrane protein in interphase and mitosis. *J. Cell. Biol.*, 1997. **138**(6): p. 1193-206.

117. Shimokawa, T. and W.L. Smith, Essential histidines of prostaglandin endoperoxide synthase. His-309 is involved in heme binding. *J. Biol. Chem.*, 1991. **266**(10): p. 6168-73.

118. Bhattacharyya, D.K., *et al.*, Selective inhibition of prostaglandin endoperoxide synthase-1 (cyclooxygenase-1) by valerylsalicylic acid. *Arch. Biochem. Biophys.*, 1995. **317**(1): p. 19-24.

119. Van Der Ouderaa, F.J., *et al.*, Acetylation of prostaglandin endoperoxide synthetase with acetylsalicylic acid. *Eur. J. Biochem.*, 1980. **109**(1): p. 1-8.

120. Lecomte, M., *et al.*, Acetylation of human prostaglandin endoperoxide synthase-2 (cyclooxygenase-2) by aspirin. *J. Biol. Chem.*, 1994. **269**(18): p. 13207-15.

121. Gierse, J.K., *et al.*, A single amino acid difference between cyclooxygenase-1 (COX-1) and -2 (COX-2) reverses the selectivity of COX-2 specific inhibitors. *J. Biol. Chem.*, 1996. **271**(26): p. 15810-4.

122. McLean, I.W. and P.K. Nakane, Periodate-lysine-paraformaldehyde fixative. A new fixation for immunoelectron microscopy. *J. Histochem. Cytochem.*, 1974. **22**(12): p. 1077-83.

123. Tokuyasu, K.T., Use of poly(vinylpyrrolidone) and poly(vinyl alcohol) for cryoultramicrotomy. *Histochem. J.*, 1989. **21**(3): p. 163-71.

124. Kargman, S., *et al.*, Characterization of Prostaglandin G/H Synthase 1 and 2 in rat, dog, monkey, and human gastrointestinal tracts. *Gastroenterology*, 1996. **111**(2): p. 445-54.

125. Otto, J.C. and W.L. Smith, The orientation of prostaglandin endoperoxide synthase-1 in the endoplasmic reticulum of transiently transfected cos-1 cells. *Adv. Prostaglandin Thromboxane Leukot. Res.*, 1995. **23**: p. 29-34.

126. Smith, W.L. and T.G. Bell, Immunohistochemical localization of the prostaglandin-forming cyclooxygenase in renal cortex. *Am. J. Physiol.*, 1978. **235**(5): p. F451-7.

127. Reiger, M.K., *et al.*, Subcellular localization of prostaglandin endoperoxide synthase-2 in murine 3T3 cells. *Arch. Biochem. Biophys.*, 1993. **301**(2): p. 439-44.

128. Shimokawa, T., *et al.*, Tyrosine 385 of prostaglandin endoperoxide synthase is required for cyclooxygenase catalysis. *J. Biol. Chem.*, 1990. **265**(33): p. 20073-6.

129. Schilling, L.J. and P.J. Farnham, Inappropriate transcription from the 5' end of the murine dihydrofolate reductase gene masks transcriptional regulation. *Nucleic. Acids Res.*, 1994. **22**(15): p. 3061-8.

130. Innis, J.W. and W.A. Scott, Chromatin structure of simian virus 40-pBR322 recombinant plasmids in COS-1 cells. *Mol. Cell Biol.*, 1983. **3**(12): p. 2203-10.

131. Hempel SL, M.M., Hunninghake GW, Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes. *J. Clin. Invest.*, 1994. **93**(1): p. 391-396.

132. Claria, J. and C.N. Serhan, Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions. *Proc. Natl. Acad. Sci.U.S.A.*, 1995. **92**(21): p. 9475-9.

133. Evett, G.E., *et al.*, Prostaglandin G/H synthase isoenzyme 2 expression in fibroblasts: regulation by dexamethasone, mitogens, and oncogenes. *Arch. Biochem. Biophys.*, 1993. **306**(1): p. 169-77.

134. Luong, C., *et al.*, Flexibility of the NSAID binding site in the structure of human cyclooxygenase-2. *Nat. Struct. Biol.*, 1996. **3**(11): p. 927-33.

135. Otto, J.C. and W.L. Smith, Photolabeling of prostaglandin endoperoxide H synthase-1 with 3-trifluoro-3-(m-[^{125}I]iodophenyl)diazirine as a probe of membrane association and the cyclooxygenase active site. *J. Biol. Chem.*, 1996. **271**(17): p. 9906-10.

136. Picot, D. and R.M. Garavito, Prostaglandin H synthase: implications for membrane

structure. *FEBS Lett.*, 1994. **346**(1): p. 21-5.

137. Tontonoz, P., E. Hu, and B.M. Spiegelman, Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor [published erratum appears in *Cell* 1995 Mar 24;80(6):following 957]. *Cell*, 1994. **79**(7): p. 1147-56.

138. Murakami, M., *et al.*, c-kit ligand mediates increased expression of cytosolic phospholipase A_2, prostaglandin endoperoxide synthase-1, and hematopoietic prostaglandin D2 synthase and increased IgE-dependent prostaglandin D2 generation in immature mouse mast cells. *J. Biol. Chem.*, 1995. **270**(7): p. 3239-46.

139. Balsinde, J., *et al.*, Arachidonic acid mobilization in P388D1 macrophages is controlled by two distinct Ca(2+)-dependent phospholipase A_2 enzymes. *Proc. Natl. Acad. Sci. U.S.A.*, 1994. **91**(23): p. 11060-4.

140. Murakami, M., Y. Nakatani, and I. Kudo, Type II secretory phospholipase A_2 associated with cell surfaces via C-terminal heparin-binding lysine residues augments stimulus-initiated delayed prostaglandin generation. J. Biol. Chem., 1996. **271**(47): p. 30041-51.

141. Kulmacz, R.J., R.B. Pendleton, and W.E. Lands, Interaction between peroxidase and cyclooxygenase activities in prostaglandin-endoperoxide synthase. Interpretation of reaction kinetics. *J. Biol. Chem.*, 1994. **269**(8): p. 5527-36.

142. Capdevila, J.H., *et al.*, The highly stereoselective oxidation of polyunsaturated fatty acids by cytochrome P450BM-3. *J. Biol. Chem.*, 1996. **271**(37): p. 22663-71.

143. Swinney, D.C., *et al.*, Differential allosteric regulation of prostaglandin H synthase 1 and 2 by arachidonic acid. *J. Biol. Chem.*, 1997. **272**(19): p. 12393-8.

144. Byrum, R.S., *et al.*, Role of the 5-lipoxygenase-activating protein (FLAP) in murine acute inflammatory responses. *J. Exp. Med.*, 1997. **185**(6): p. 1065-75.

145. Abramovitz, M., *et al.*, 5-lipoxygenase-activating protein stimulates the utilization of arachidonic acid by 5-lipoxygenase. *Eur. J. Biochem.*, 1993. **215**(1): p. 105-11.

146. Kargman, S., P.J. Vickers, and J.F. Evans, A23187-induced translocation of 5-lipoxygenase in osteosarcoma cells. J. Cell Biol., 1992. **119**(6): p. 1701-9.

147. Vickers, P.J., 5-Lipoxygenase-activating protein (FLAP). *J. LipidMediat. Cell Signal.*, 1995. **12**(2-3): p. 185-94.

148. Soullam, B. and H.J. Worman, The amino-terminal domain of the lamin B receptor

is a nuclear envelope targeting signal. J. Cell Biol., 1993. **120**(5): p. 1093-100.

149. Song, I. and W.L. Smith, C-terminal Ser/Pro-Thr-Glu-Leu tetrapeptides of prostaglandin endoperoxide H synthases-1 and -2 target the enzymes to the endoplasmic reticulum. *Arch. Biochem. Biophys.*, 1996. **334**(1): p. 67-72.

150. Wang, X., *et al.*, Purification of an interleukin-1 beta converting enzyme-related cysteine protease that cleaves sterol regulatory element-binding proteins between the leucine zipper and transmembrane domains. *J. Biol. Chem.*, 1995. **270**(30): p. 18044-50.

151. Clark, J.D., *et al.*, A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca(2+)-dependent translocation domain with homology to PKC and GAP. *Cell,* 1991. **65**(6): p. 1043-51 issn: 0092-8674.

152. Channon, J.Y. and C.C. Leslie, A calcium-dependent mechanism for associating a soluble arachidonoyl-hydrolyzing phospholipase A_2 with membrane in the macrophage cell line RAW 264.7. *J. Biol. Chem.*, 1990. **265**(10): p. 5409-13.

153. Smith, W.L., Prostaglandin biosynthesis and its compartmentation in vascular smooth muscle and endothelial cells. *Annu. Rev. Physiol.*, 1986. **48**: p. 251-62.

154. Ruan, K.H., *et al.*, Amino-terminal topology of thromboxane synthase in the endoplasmic reticulum. J. Biol. Chem., 1993. **268**(26): p. 19483-90.

155. Blobel, G., Intracellular protein topogenesis. *Proc. Natl. Acad. Sc.i U.S.A.*, 1980. **77**(3): p. 1496-500.

156. Brunner, J. and G. Semenza, Selective labeling of the hydrophobic core of membranes with 3-(trifluoromethyl)-3-(m-[125I]iodophenyl)diazirine, a carbene-generating reagent. *Biochemistry*, 1981. **20**(25): p. 7174-82.

157. Blanton, M.P. and J.B. Cohen, Identifying the lipid-protein interface of the Torpedo nicotinic acetylcholine receptor: secondary structure implications. *Biochemistry,* 1994. **33**(10): p. 2859-72.

158. DeWitt, D.L., *et al.*, Orientation of the active site and antigenic determinants of prostaglandin endoperoxide synthase in the endoplasmic reticulum. *J. Biol. Chem.*, 1981. **256**(20): p. 10375-82.

159. Movileanu, L., *et al.*, Selective association of phospholipids as a clue for the passive flip-flop diffusion through bilayer lipid membranes. *Biosystems*, 1997. **40**(3): p. 263-75.

160. Paris, S., *et al.*, Role of protein-phospholipid interactions in the activation of ARF1 by the guanine nucleotide exchange factor Arno. *J. Biol. Chem.*, 1997. **272**(35): p. 22221-6.

161. Morrissey, J.H., *et al.*, Factor VIIa-tissue factor: functional importance of protein-membrane interactions. *Thromb. Haemost.*, 1997. **78**(1): p. 112-6.

162. Haverstick, D.M. and M. Glaser, Visualization of Ca2+-induced phospholipid domains. *Proc. Natl. Acad. Sci. U.S.A.*, 1987. **84**(13): p. 4475-9.

163. Hempel SL, Monick MM, He B, Yano T, Hunninghake GW, Synthesis of prostaglandin H synthase-2 by human alveolar macrophages in response to lipopolysaccharide is inhibited by decreased cell oxidant tone. *J. Biol. Chem.*1994, **269**:52, p. 32979-32984.

164. Hempel SL, Monick MM, Hunninghake GW, Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes. *J. Clin. Invest.*, 1994, **93**:1, p. 391-396.

165. Hoff, T., DeWitt, D., Kaever, V., Resch, K.,Goppelt-Struebe, M. Differentiation-associated expression of prostaglandin G/H synthase in monocytic cells. 1993, *FEBS Lett.*, **320**:1, p. 38-42.

166. Funk, C.D., Furci, L., FitzGerald, G.A., *et al.*, Point mutatioin in the seventh hydrophobic domain of the human thromboxane A_2 receptor allows discrimination between agonist and antagonist binding sites. 1993, *Mol. Pharmacol.* **4**, 934-939.

167. Sugimoto, Y., Yamasaki, A., *et al.* Failure of Parturition in Mice Lacking the Prostaglandin $F_{2\alpha}$ Receptor. 1997, *Science* **277**: 681-683.

168. Tanaka, T., *et al.* Effects of growth temperature on the fatty acid composition of the free-living nematode Caenorhabditis elegans. 1996, *Lipids* **31**:11, 1173-1178.