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Subcellular Localization of PGH Synthase-1 and -2, and Cytosolic Phospholipase A_2

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SUBCELLULAR LOCALIZATION OF PGH SYNTHASE-1 AND -2, AND CYTOSOLIC PHOSPHOLIPASE A_2

By

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ABSTRACT

SUBCELLULAR LOCALIZATION OF PGH SYNTHASE-1 AND -2, AND CYTOSOLIC PHOSPHOLIPASE A_2

By

Martha Kay Regier

PGH synthase-1 and -2 catalyze the conversion of arachidonic acid to prostaglandin H_2, the precursor for synthesis of biologically active prostanoids. One source of substrate arachidonic acid is provided by cytosolic phospholipase A_2 hydrolysis of membrane phospholipids. Using immunocytochemistry and confocal microscopy we have investigated several aspects of PGH synthase-1 and -2 and cytosolic phospholipase A_2 subcellular localization.

The C-terminal sequence of PGH synthase-1 and-2 from a variety of species is -P/STEL, which is similar to the C-terminal sequences of a number of proteins which are localized to the endoplasmic reticulum by the -KDEL retention system. We tested the hypothesis that -P/STEL was the ER retention signal for PGH synthase. Mutants in ovine PGH synthase-1 that would disrupt the putative -PTEL retention signal were transiently transfected in *cos*-1 cells and analyzed by immunocytochemistry. Mutation of -PTEL did not alter localization or cause secretion of PGH synthase. We conclude that PGH synthase-1 localization is independent of its C-terminal tetrapeptide.

Immunocytochemistry was used to determine the subcellular localization of PGH synthase-2 in NIH 3T3 fibroblasts. Isozyme selective antisera showed that PGH synthase-2 in serum stimulated fibroblasts is localized to the same membranes as PGH synthase-1, the endoplasmic reticulum and nuclear envelope. Additional work from our laboratory showed that PGH synthase-2 is

concentrated at the nuclear envelope. We postulated that the 18 amino acids near the C-terminus of PGH synthase-2 (PGHS-2 cassette) could function as a PGH synthase-2 nuclear envelope localization domain. A PGH synthase-2 mutant that lacks the PGHS-2 cassette was generated. This mutant retains cyclooxygenase and peroxidase activity and has a K_m similar to native PGH synthase-2, and is a useful tool for future localization studies.

Cytosolic phospholipase A_2 ($cPLA_2$) is known to translocate from the soluble to the particulate fraction of cells in a Ca^{2+} dependent mechanism. We determined that translocated $cPLA_2$ is localized to the nuclear envelope and endoplasmic reticulum. Analysis of two $cPLA_2$ mutants showed that the capacity to translocate to these membranes was dependent on the Ca^{2+} dependent phosphoslipid binding domain and independent of phosphorylation by MAP kinase. Using the same mutants we determined that $cPLA_2$ liberation of arachidonate is dependent on phosphorylation *and* the calcium dependent phospholipid binding domain.

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LIST OF ABBREVIATIONS

5-LO	5-lipoxygenase
BiP	immunoglobulin heavy chain binding protein
CaLB	calcium-dependent phospholipid binding
cPLA$_2$	cytosolic phospholipase A2
DME	Dulbecco's modified Eagle medium
EGF	epidermal growth factor
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
IL	interleukin
K_m	Michaelis-Menton constant
MAP kinase	mitogen-activated protein kinase
NE	nuclear envelope
NE/ER ratio	quantitated ratio of nuclear envelope to endoplasmic reticulum fluorescence intensity
NSAID	non-steroidal anti-inflammatory drug
PBS	phosphate-buffered saline
PDGF	platelet derived growth factor
PG	prostaglandin
PGH	prostaglandin endoperoxide H
PLA2	phospholipase A2
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TLC	thin-layer chromatography
TX	thromboxane
V_{max}	maximum velocity of a reaction

CHAPTER 1

LITERATURE REVIEW

PART 1: PGH SYNTHASE

Introduction and Chapter Overview

Biological processes in animal systems depend on a variety of signaling molecules to communicate between cells, tissues and organ systems. One class of signaling molecules is derived from fatty acids, predominately arachidonic acid (all *cis* 5,8,11,14-eicosatetraenoic acid). Arachidonic acid is a common precursor for metabolism by three enzyme systems, the cyclooxygenase, lipoxygenase and epoxygenase pathways. These enzyme systems, intermediate structures and final products make up the arachidonate cascade (Figure 1). Products from these pathways constitute a group of oxygenated, 20-carbon unsaturated fatty acids with biological signaling activity. Collectively they are known as eicosanoids, and they initiate a variety of cell-specific biological responses involved in a broad range of basic physiological processes. The three pathways of the arachidonate cascade are distinguished by the manner in which the respective enzyme systems introduce oxygen into the fatty acid, and in the structure and biological effect of these products. The lipoxygenase pathway is reviewed in reference 1; and the epoxygenase pathway in reference 2. This chapter will focus on PGH synthase, the central enzyme of the cyclooxygenase pathway. In Part 1: PGH synthase I review the physiological effects and biosynthesis of prostaglandins (PGs), PGH synthase isozyme regulation, and biochemical characterization of

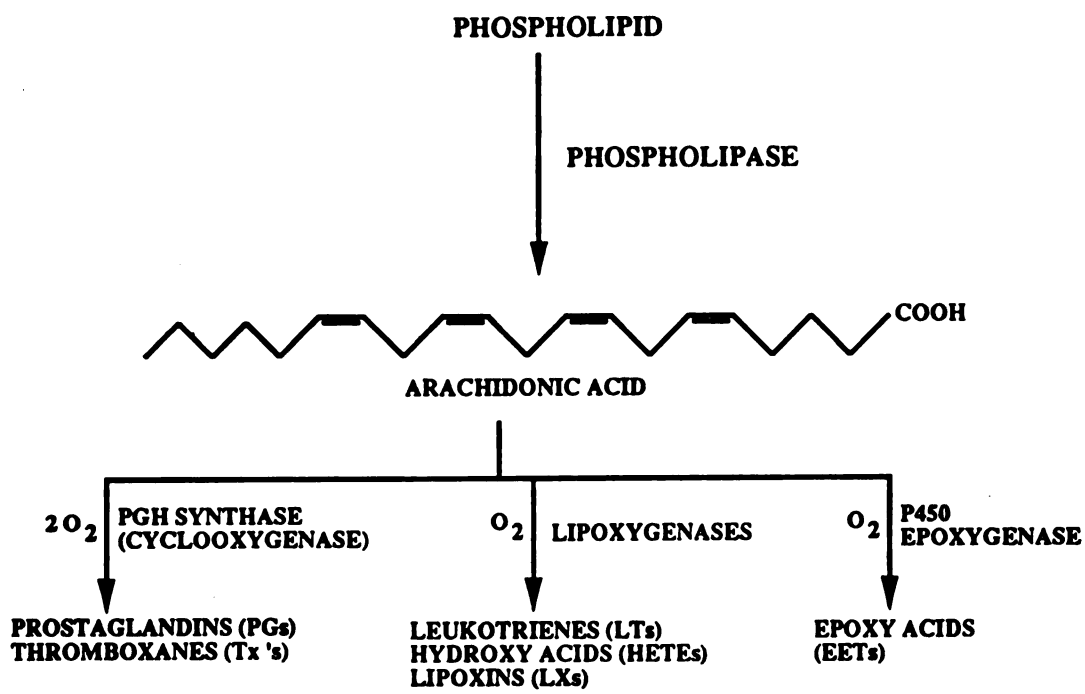


Figure 1. The arachidonate cascade. Arachidonate is metabolized by three different enzyme systems: the cyclooxygenase, lipoxygenase, and P450 expoxygenase pathways. Oxygenated derivatives of arachidonate are the products of these pathways.

PGH synthase, with an emphasis on protein structure and topology. In Part 2: Protein Targeting I review several mechanisms of intracellular protein targeting, which relates to my studies of PGH synthase subcellular localization.

Physiological action of prostaglandins

Prostaglandins and thromboxane, collectively called prostanoids, are products of the cyclooxygenase pathway. They are produced in virtually all animal tissues, but not necessarily in all cell types of the tissue (3), and play a role in a broad range of basic physiological events (4-6). Prostanoids are synthesized in response to extracellular stimuli such as circulating hormones, growth factors and cytokines (4,7). Generally they work as local hormones to coordinate and modulate cellular responses to multiple stimuli in the tissue. For example, prostanoids play an important role in the regulation of vascular homeostasis. In response to a number of effectors including thrombin, bradykinin, and interleukin-1 (IL-1), arterial endothelial cells produce prostacyclin (PGI_2), a vasodilator and inhibitor of platelet aggregation (8). Thrombin stimulated platelets produce thromboxane A2 (TXA_2), a vasoconstrictor and platelet aggregator (9,10). Together PGI_2 and TXA_2 work in opposition to each other within the context of a much more complex biochemical regulatory system to maintain vascular homeostasis and blood flow. Prostaglandins also play a role in diverse physiological events such as gastric acid secretion in the stomach, ovulation, inflammation at sites of injury, and water reabsorption in the kidney (4,7,11,12).

Prostaglandin signaling

Prostanoids exert their actions by generating second messenger signals. After synthesis, prostanoids exit the cell by an undetermined pathway and interact with prostanoid receptors on the surface of the synthesizing cell or neighboring cells (Figure 2) (6,7). Prostanoid receptors are members of the heterotrimeric G protein-linked superfamily of receptors. G proteins couple receptors to various intracellular effector systems including adenylate cyclase, phospholipase C, and Ca^{2+}. For example, PGE_2 receptors are divided into four subtypes based on their coupling characteristics. EP1 is coupled to increases in Ca^{2+}; EP2 and EP4 to activation of adenylate cyclase; and EP3, to phosphatidylinositol turnover and modulation of adenylate cyclase (13). In the kidney PGE_2 acts in cells of the collecting tubule and thick ascending limb to help regulate water reabsorption. The EP2 receptor is coupled positively to adenylate cyclase to increase cAMP concentrations. This action of PGE_2 augments the stimulus of circulating anti-diuretic hormone. PGE_2 also interacts with an adenylate cyclase inhibitory receptor (EP3) which decreases cAMP concentrations and attenuates the hormonal stimulus (7).

Prostaglandins work within the context of complex multi-agonist signaling pathways to modulate or coordinate cellular responses. The great diversity of processes in which prostaglandins are involved is a function of many points of cell and tissue specificity—the synthesis of a specific prostanoid species within a cell, the expression of appropriate prostaglandin receptors in the same and neighboring cells, the type of effector coupled to the receptor and the positive or negative modulation of the resulting second messenger levels, and the translation of this signal into cell or tissue-specific action.

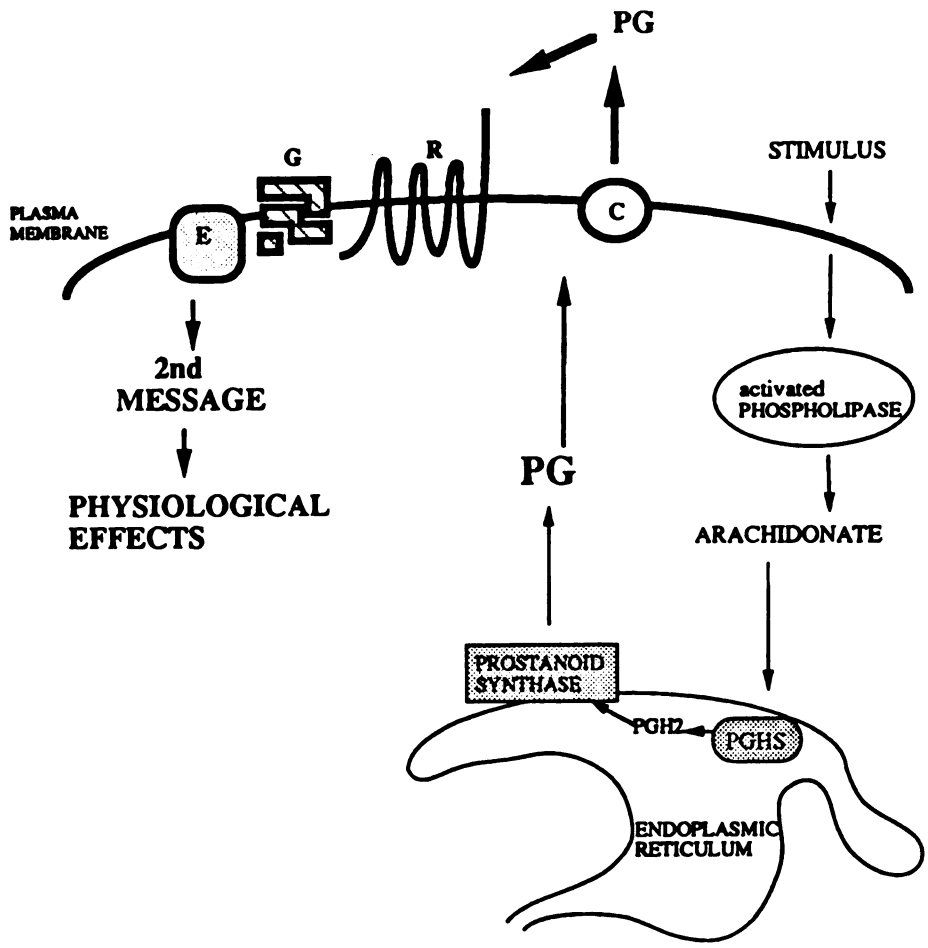


Figure 2. Prostaglandin signaling pathway. Prostaglandins (PGs) are synthesized in response to extracellular stimuli. After synthesis PG exits the cell presumably via a carrier protein (C). Extracellular PG binds to its cognate cell surface receptor (R) and acts as a local hormone to generate intracellular second messengers. This signaling pathway is mediated by the G protein (G) coupled receptor and the associated effector enzyme (E).

Prostanoid synthesis

Formation of prostanoids occurs in three steps: mobilization of arachidonate substrate, conversion of arachidonate to PGH_2 by PGH synthase, and conversion of PGH_2 to a specific prostanoid by cell-specific prostanoid synthases. This sequence of events is depicted in Figure 3.

Mobilization of arachidonate acid for prostanoid synthesis

Arachidonate, an ω–6 essential fatty acid, is provided to cells by the uptake of dietary fatty acids and is then incorporated into membrane lipids (14). In this way PGH synthase substrate is 'stored' in cellular membranes, esterified at the *sn*-2 position of glycerophospholipids, and is released when phospholipases hydrolyze phospholipids. Because intracellular levels of free (unesterified) arachidonate are low (15-17), liberation of the substrate is an important point of regulation for prostanoid synthesis. Extracellular stimuli which activate phospholipases include circulating hormones, inflammatory cytokines and growth factors (18). Activation occurs through interaction of the stimulus with its cognate cell surface receptor and subsequent downstream signaling pathways that are not well understood. Arachidonate can be derived from phospholipids by a variety of coordinated phospholipase and lipase activities, and it is not certain which pathways are most significant *in vivo* (16-18).

One pathway for the mobilization of arachidonate is by direct action of phospholipase A_2 (PLA_2), which cleaves fatty acids at the *sn*-2 position of phospholipids (18). PLA_2 activity is found in a group of enzymes which differ in structural and biochemical characteristics . Cytosolic type PLA_2 ($cPLA_2$) (Mr=85 kD) is thought to play a major role in arachidonate release because it preferentially cleaves arachidonate-containing phospholipids, and is activated

Figure 3. Biosynthetic pathway for prostanoid synthesis. Adapted from Smith, W. L., 1992 (6).

PATHWAY FOR PROSTANOID SYNTHESIS

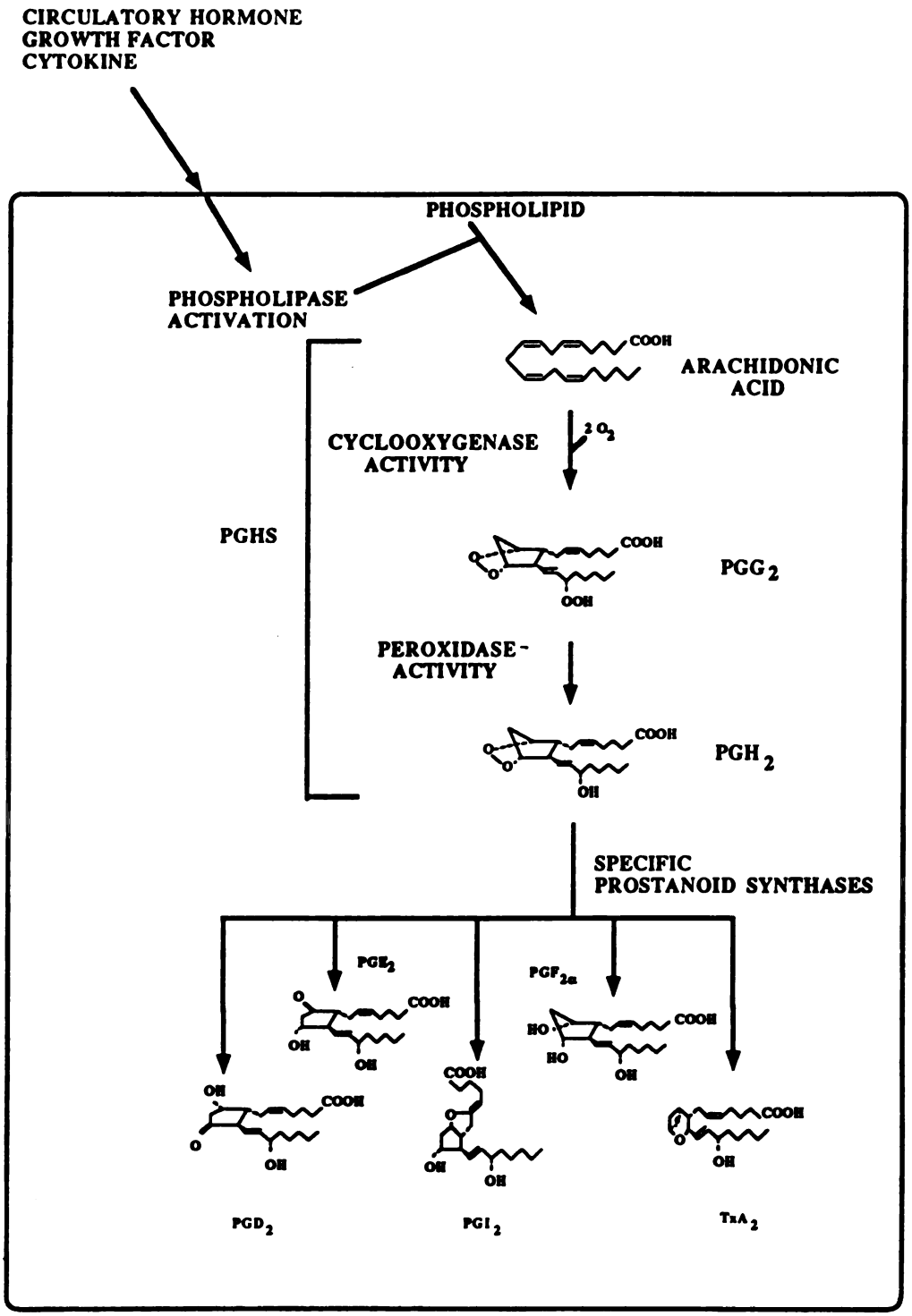


FIGURE 3

by submicromolar Ca^{2+} levels achieved in stimulated cells (18-21). In the process of activation, cPLA2 is phosphorylated (22), and translocates from a soluble fraction to a particulate membrane fraction (20). Indirect fluorescence immunocytochemistry and confocal microscopy shows that cPLA2 is translocated from the cytosol to the nuclear envelope (NE) and the endoplasmic reticulum (ER), but not to the plasma membrane (See chapter 5). It seems plausible that in stimulated cells physical juxtaposition of cytosolic PLA2 and PGH synthase in the endoplasmic reticulum and NE could facilitate aspects of substrate-enzyme interaction.

A second PLA_2 enzyme, secretory type II PLA_2 (sPLA2) (Mr=14 kDa), may also release arachidonate for prostaglandin synthesis. sPLA2 lacks fatty acid specificity and requires millimolar Ca^{2+} concentrations for activity (18,23), unlike $cPLA_2$. Because the primary structure of $sPLA_2$ reveals a typical signal peptide for secretion and lacks additional targeting signals, the enzyme is most likely a secreted protein (18). High levels of $sPLA_2$ are found in extracellular environments of inflammation such as synovial fluid of patients with rheumatoid arthritis (24). This observation, in conjunction with high levels of prostaglandins at inflammatory sites, suggests that arachidonate released by $sPLA_2$ may be converted to prostanoids in inflammatory events.

Other pathways can also release arachidonate. Phospholipase C generates diacylglycerol that is hydrolyzed by di- or monoacylglycerolipases to yield arachidonate (25,26). Exogenous sources may directly contribute arachidonate; the low density lipoprotein (LDL) receptor pathway supplies cells with arachidonate presumably from arachidonyl cholesterol ester in LDL (27,28).

Release of arachidonate in a biphasic pattern has been observed in platelet-derived growth factor (PDGF) stimulated Swiss 3T3 cells (29). The first phase

occurs within 15 minutes of stimulation by action of cytosolic PLA_2. The second phase is sustained for over 2 hours, and accounts for over 90% of the total arachidonate released. However, this release is not attributable to $cPLA_2$ activity and is dependent on protein synthesis. This suggests that mechanisms other than immediate action of $cPLA_2$ provide additional pathways for arachidonate release. Interestingly, a biphasic synthesis of prostaglandins has also been observed in PDGF-treated 3T3 cells (30). Recent reports also suggest that the arachidonate for prostaglandin synthesis is stored in PGH synthase isozyme selective pools that are mobilized according to isozyme specific extracellular signals (31,32).

PGH synthase enzyme activity—Synthesis of PGH_2

Free arachidonate is converted to PGH_2 by PGH synthase. PGH synthase has two catalytic activities, a cyclooxygenase activity and a peroxidase activity. The cyclooxygenase activity adds two molecules of oxygen to arachidonate. The cyclooxygenase reaction is initiated by abstraction of the pro-S hydrogen at position C-13 (33) which is mediated through a nearby tyrosyl radical. Then one oxygen molecule is added to the C-11 position where it further reacts to form an endoperoxide bridge to the C-9 position. A second oxygen is added to the C-15 position forming the hydroperoxide product PGG_2 (33). The peroxidase activity subsequently reduces the hydroperoxide at position C-15 to a hydroxyl group to produce PGH_2 (33). Residues important for catalysis are Tyr385, the putative source of the tyrosyl radical involved in the cycloxygenase reaction (34), and His388 and His209 which coordinate the required heme molecule (35,36) (Figure 4).

There are two forms of PGH synthase (6), PGH synthase-1 and PGH synthase-2, and they are biochemically similar. Both enzymes catalyze the

OVINE PGH SYNTHASE-1 ACTIVE SITE

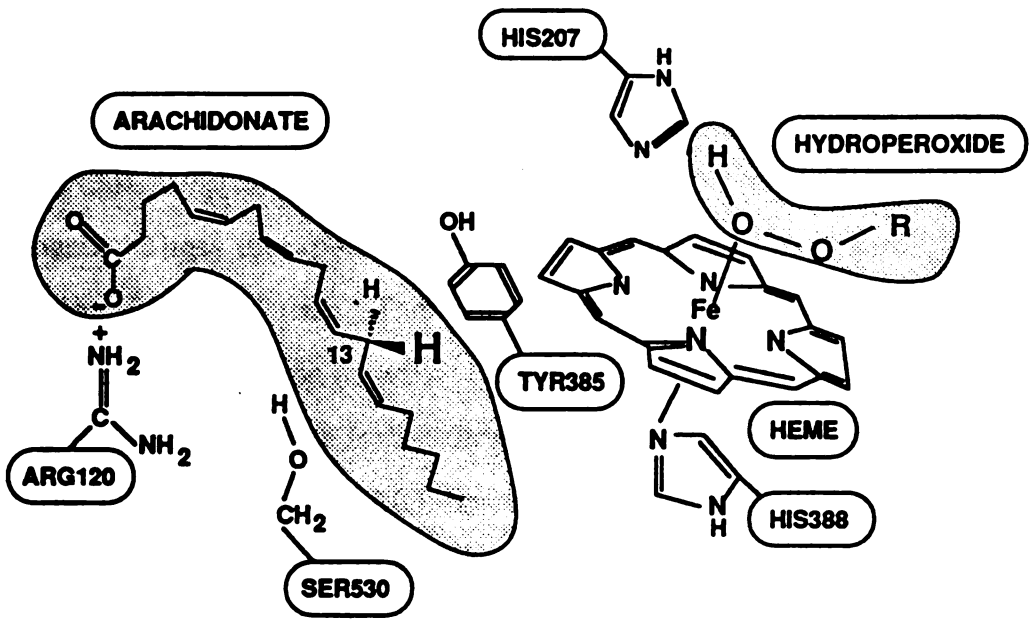


Figure 4. Model for the active site for ovine PGH synthase-1. Adapted from Smith and Laneuville, 1994 (37).

same cyclooxygenase and peroxidase reaction (38) and have similar values for K_m and V_{max} (39-41). The residues important for catalysis are also conserved (6). The regulation and biochemical characterization of these isozymes are considered later in this chapter.

Inhibition of PGH synthase by NSAIDs

The cyclooxygenase activity of PGH synthases is inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) (37,42-44). The fact that the peroxidase activity of this enzyme is not inhibited by these agents provided early evidence that the cyclooxygenase and peroxidase reactions occurred at distinct active sites (33). Typical NSAIDs include aspirin, ibuprofen and indomethacin. These therapeutic agents are administered to reduce inflammation, fever and pain. These effects result principally from NSAID inhibition of cyclooxygenase activity. NSAIDs inhibit PGH synthase by competing with arachidonate for binding to the cyclooxygenase active site. Aspirin, the most common NSAID, has an unique inhibitory mechanism: it covalently modifies the protein by acetylation of serine 530 (45) (46). The modified serine is located in the substrate binding channel (36), and steric exclusion by the bulky acetyl group is thought to preclude substrate binding (47) (46).

NSAIDs inhibit both PGH synthase isozymes (45,46). The anti-inflammatory action of NSAIDs is likely due to inhibition of PGH synthase-2, the isozyme implicated in prostaglandin synthesis of inflammatory events (48,49). Likewise, the gastric ulcers that accompany chronic administration of NSAIDs may be due to inhibition of PGH synthase-1, the isozyme implicated in maintenance of physiological homeostasis (48). The PGH synthase isozymes are pharmacologically distinct (37,39), and there is intense interest

in identifying isozyme-selective NSAIDs. Recently PGH synthase-1 (50) and -2 selective inhibitors have been isolated (51).

Conversion of PGH_2 to specific prostanoids

PGH_2 is the common precursor for the formation of specific prostanoids by prostanoid synthases that catalyze specific isomeric rearrangements (5,6,33). The five or six membered aromatic ring of the prostaglandins and thromboxanes is a unique structural feature of these cyclooxygenase pathway products. Which prostanoid is synthesized in a cell depends on the particular prostanoid synthase expressed in that cell. Generally a given cell synthesizes only one prostaglandin or thromboxane (5,33). However, most cells express multiple prostanoids receptors, so that they are responsive to multiple prostanoid signals (6).

PGH synthase isozymes and their regulation

Two forms of PGH synthase have been identified in mammalian cells. PGH synthase-1 was first purified in 1976 from sheep seminal vesicles (52,53), an unusually rich source of the enzyme. The cDNA encoding PGH synthase-1 was initially cloned from ovine cDNA libraries (54-56). PGH synthase-2 was discovered in 1991 (57-59). The PGH synthase-2 cDNA was then cloned in 1991 from mouse (58) and chicken (57). A complete complement of cDNAs for both isozymes have been isolated from mouse (45,58), human (60-62), and rat (63,64).

The two isozymes catalyze the same reactions with similar kinetic parameters to form PGH_2 (43,58), and they exhibit many biochemical similarities. However, in terms of their pattern of expression they are dissimilar. PGH synthase-1 is constitutively expressed, whereas PGH

synthase-2 is expressed transiently following stimulation of the cell by growth factors, cytokines, and tumor promoters (65). Much of the work describing expression of PGH synthase and prostaglandin synthesis was carried out prior to identification of a second PGH synthase isozyme, and therefore many of these observations must be reexamined with isozyme selective tools to distinguish the roles of each. Below is an general summary of what is currently understood about the regulation of these enzymes.

Regulation of PGH synthase-2

PGH synthase-2 was originally identified serendipitously in laboratories studying the regulation of gene expression by oncogenes and mitogenic stimuli (57-59). Expression of the gene is dramatically induced by a variety of stimuli including serum, platelet-derived growth factor (PDGF), epidermal growth factor, endothelin, interleukin-1, lipopolysaccharide (LPS), phorbol esters, calcium ionophores, and forskolin (65). When stimulated with the appropriate agent(s), induction of PGH synthase-2 expression is observed in a variety of cell types including fibroblasts, monocytes, and epithelial cells (65). For example, expression of PGH synthase-2 in fibroblasts is induced by serum, PDGF and phorbol ester, in macrophage by LPS, and in ovarian granulosa cells by human chorionic gonadotropin. These ligands induce PGH synthase-2 through a complex network of signaling pathways that are likely to depend on the particular cell system. In rat mesangial cells expression of PGH synthase-2 by serotonin (66,67) or endothelin (66) is stimulated through a tyrosine kinase signaling pathway.

Expression of PGH synthase-2 mRNA in response to cell stimulation is rapid and transient and is augmented (super induced) by treatment with cycloheximide (68,69). These features are hallmarks of a group of genes called

'immediate early genes' or primary response genes. Immediate early genes are those genes which are immediately up-regulated in response to growth stimulating factors like PDGF, and inflammatory signals like IL-1, and are proposed to play a central role in events leading to cell growth (70,71).

In Swiss and NIH 3T3 murine fibroblasts expression of PGH synthase has been well characterized (68,72). Following treatment of such cells with serum, the rate of transcription of the PGH synthase-2 gene is increased, and corresponding increases in mRNA and protein levels follow temporally (68). PGH synthase-2 protein levels are maximal at 2-3 hours post serum stimulation and decline to near basal levels within 8 hours (68). Decreases in the rate of transcription and mRNA levels precede as expected. The mechanism for the rapid decline mRNA levels and protein has not been determined. Multiple AUUUA repeats in the 3' untranslated region of the gene are likely to destabilize the message (73). In addition it is possible that degradation of PGH synthase-2 protein is accelerated during this time, perhaps by concomitant induction of a PGH synthase-2 protease or inhibitor. Whatever the mechanisms, it is clear that PGH synthase-2 expression is tightly regulated. Expression of PGH synthase-2 in other cell types by other ligands shows a similar pattern, although the absolute time course of induction and decay vary (72)(71,73,69,70,75).

PGH synthase-2 is induced by inflammatory factors in pro-inflammatory cells. Human chorionic gonadotropin treated rat ovaries (64), LPS treated macrophages (64,74,75), and IL-1 treated fibroblasts and endothelial cells (76) transiently express PGH synthase-2. Expression of PGH synthase-2 is negatively regulated by anti-inflammatory glucocorticoids such as dexamethasone, by inhibiting transcription of the gene (68,69,72,77). Taken together, the induction of PGH synthase-2 by pro-inflammatory signals in a

pattern of immediate early genes and the negative regulation of PGH synthase-2 by anti-inflammatory glucocorticoids implicate PGH synthase-2 as the 'inflammatory' PGH synthase.

Regulation of PGH synthase-1

PGH synthase-1 is expressed constitutively in growing and quiescent cells (3,78,79). Serum stimulation of murine fibroblasts does not induce further expression of the gene, nor does glucocorticoid treatment down regulate its expression (38,68,69). In general PGH synthase-1 is expressed at constant levels. Constitutive expression of PGH synthase affords a pathway for immediate prostaglandin synthesis in response to acute, rapid response signaling needs, a capability presumably necessary for the continuous maintenance of physiological homeostasis processes. Consequently, PGH synthase-1 has been postulated to be responsible for prostaglandin synthesis for 'housekeeping functions.' PGH synthase-1 is expressed in certain cell-types within a given tissue, although the mechanism of this spatial regulation in particular cells for constitutive prostaglandin synthesis is not clear. Several reports demonstrate that PGH synthase-1 expression is activated in the course of cell development or differentiation in mononuclear phagocytes (80), smooth muscle and endothelial cells (81), and bone marrow derived mast cells (31).

Structural and biochemical properties of PGH synthase isozymes

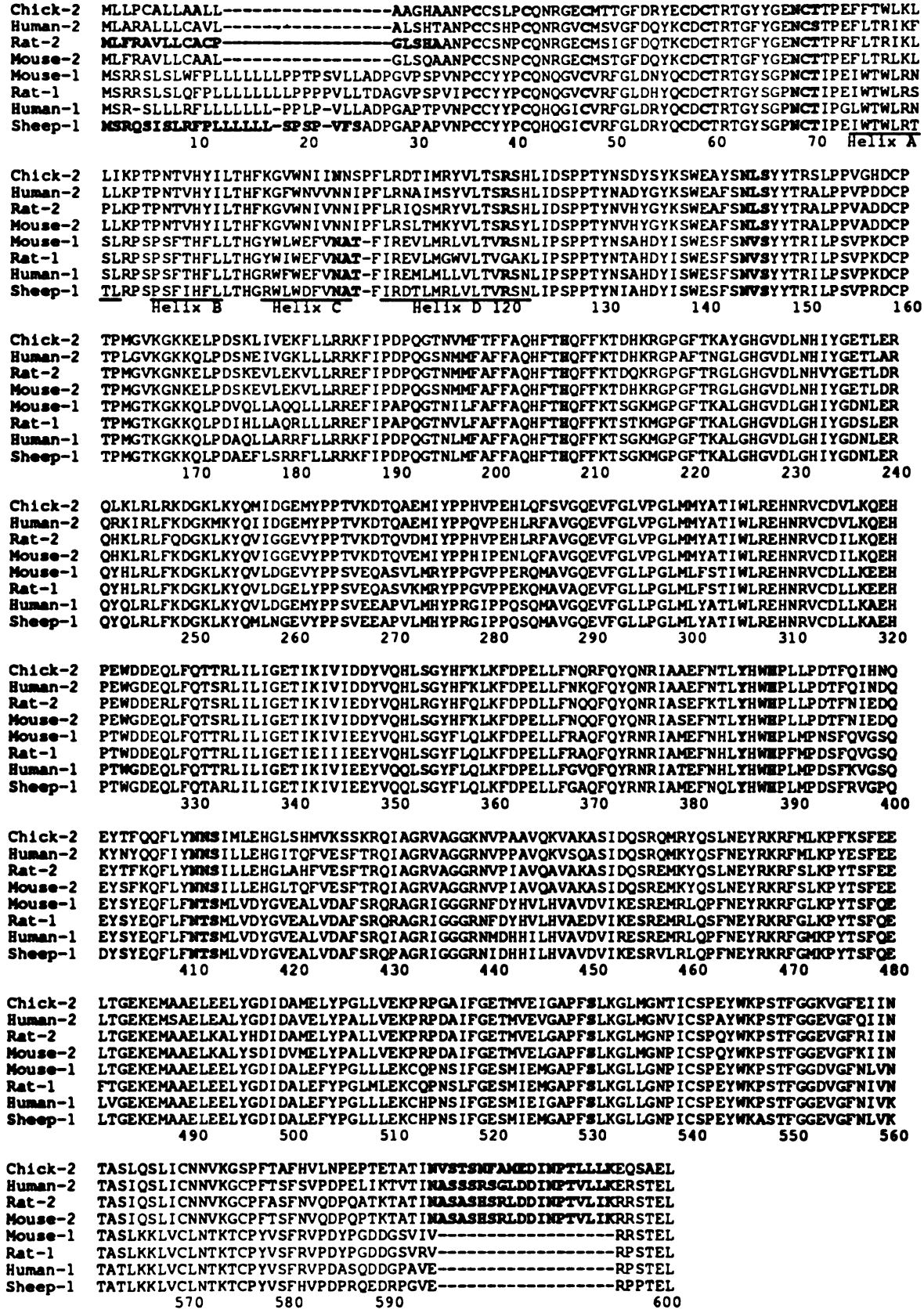
Biochemical characterization shows that PGH synthase is a heme-containing glycoprotein which can only be solubilized from cellular membrane preparations by treatment with detergent (52,53). Based on the requirement of detergent for solubilization, PGH synthase is classified as an

integral membrane protein. Data that suggests that the protein possess transmembrane domains was reported previously (55,82,83). However, in view of more recent experiments (84) and the elucidation of the PGH synthase crystal structure (36) the enzyme is better described as a integral membrane protein that is tightly associated with the membrane, but lacks membrane spanning domains. The enzyme is modified by N-linked glycosylation with carbohydrates of the high mannose form (84,85). Lack of further modification of the carbohydrates suggests that the protein is not processed by or localized in membranes beyond the cis-Golgi (86). By SDS PAGE separation, PGH synthase-1 has an apparent Mr of 72 kDa, but the enzyme exists as a non-covalent homodimer of 124 kDa in detergent solubilized membrane extracts (53,87). PGH synthase-2 separates as a 72 and 74 kDa doublet due to differential glycosylation (84).

In the course of purification PGH synthase can lose catalytic activity as a consequence of heme dissociation (53), although addition of heme reconstitutes activity. In the absence of heme, trypsin proteolysis of ovine PGH synthase-1 yields two protein fragments, 33 and 38 kDa, but PGH synthase remains intact in the presence of heme. This suggests that heme binding causes a conformational change in the protein. Resistance to trypsin digestion has been used to detect changes in protein conformation in PGH synthase mutants (47,84).

PGH synthase cDNAs have been isolated from a number of species (Figure 5): PGH synthase-1 from sheep (54-56), human (60), rat (63) and mouse (45); and PGH synthase-2 from human (61,62), rat (63,64), mouse (58) and chicken (57). Figure 5 shows an alignment of the deduced amino acid sequences of these enzymes. The sequences of PGH synthase-1 and -2 are highly conserved, with a similarity of approximately 75% depending on the species.

Figure 5. Amino acid sequences of various PGH synthases. The deduced amino acid sequences of various PGH synthases-1 and -2 are aligned for comparison: sheep PGH synthase-1 (54-56), mouse PGH synthases -1 (45) and -2 (58), human PGH synthases -1 (60) and -2 (61,62), rat PGH synthases -1 (63) and -2 (63,64), and chicken PGH synthase-2 (57). Representative signal peptides for the two isozymes are shown in bold. Other residues shown in bold: the tripeptide consensus sequences of residues modified by N-linked glycosylation Asn104, Asn144, and Asn 410; residues important in catalysis, Tyr385, His388 and His207; and the site of aspirin acetylation, Ser530. The C-terminal 18 amino acid peptide unique to PGH synthase-2 isozymes is in bold. The residues of helices predicted to form a membrane binding domain (36) are underlined.


Chick-2
Human-2
Rat-2
Mouse-2
Mouse-1
Rat-1
Human-1
Sheep-1
Helix A
Helix B
Helix C
Helix D
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130
140
150
160
170
180
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200
210
220
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FIGURE 5

Residues important in catalysis are conserved: Tyr385, His388, and His207. Serine 530, the site of acetylation of ovine PGH synthase-1 by aspirin, is conserved.

Three of the four N-linked glycosylation consensus sequences are modified in PGH synthase-1: Asn68, Asn144, and Asn410 (84). Expression of the enzyme in the presence of tunicamycin, an inhibitor of glycosylation, yields inactive enzyme; but deglycosylation of mature protein by endoglycosidase H does not cause loss of activity. This suggests that the carbohydrate structures play a role in protein folding to reach an active protein conformation, but not in enzyme catalysis. An additional glycosylation consensus sequence exists in PGH synthase-2 at N580 near the C-terminus. Glycosylation at this position is variable. Approximately 50% of the protein receives the additional glycosylation (84) and this accounts for the PGH synthase doublet separated on SDS PAGE. The mechanism and significance of this phenomenon is not known.

The extensive structural similarity of the isozymes diverges at the amino- and carboxy-terminal regions. At the N-terminus the isozymes have signal peptides of dissimilar sequence and length. Moreover, PGH synthase-2 contains an 18 amino acid sequence at the C-terminus that is not present in PGH synthase-1. This unique peptide shows no sequence similarity with genes reported in the GenBank.

PGH synthase crystal structure

Recently, Garavito and colleagues reported the crystal structure of ovine PGH synthase-1 (36). The structure has a high helical content. The overall globular shape is divided into three folding units: an epidermal growth factor-like (EGF) domain, a membrane binding domain, and a catalytic

domain (Figure 6). There are no apparent transmembrane domains. Structural information for the last 15 C-terminal residues was not obtained which suggests that this region of the protein has no well-defined structure.

EGF-like domains are a common protein structural motif involved in protein-protein interactions (88). PGH synthase-1 residues 25 to 70 encode an EGF-like domain which is hypothesized to function in PGH synthase dimerization. The crystal structure shows this EGF domain has extensive contact with the adjacent monomer, in a head to tail configuration, which supports this idea. Because the enzyme active sites do not appear physically related to the protein regions of dimerization in the crystal structure, it is not clear how dimerization is important for enzyme activity.

The catalytic domain is composed of two adjacent but spatially distinct active sites. The cyclooxygenase site is formed by a long hydrophobic channel which passes to the center of the protein from the exterior. Important residues in the channel include Ser530, Arg120 and Tyr385. Acetylation of Ser530 by aspirin is expected to block substrate from entering the channel. Tyrosine 385 is located at the top of the channel in a position consistent with its putative role as a tyrosyl radical which abstracts the hydrogen from C-13 of arachidonate. Arg120 is located in position for ionic interaction with the arachidonate carboxy-terminus. The peroxidase active site is located nearly opposite the cyclooxygenase active site. The heme is coordinated by His207 and His388, and is separated from but in close proximity to Tyr385 of the cyclooxygenase active site. PGG_2 dissociates from the enzyme prior to being converted to PGH_2.

The crystal structure lacks transmembrane domains which is surprising based on earlier topology data (55,82-84). Four nearly co-planar, amphipathic helices, residues 73 to 116, are postulated by Garavito and coworkers to

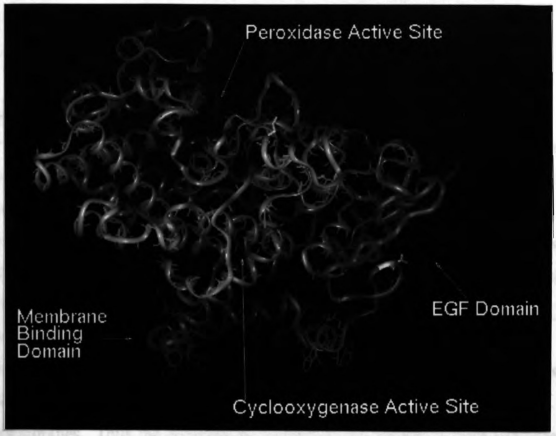

Figure 6. Crystal structure of ovine PGH synthase-1. Crystal structure coordinates were generously provided by R. Michael Garavito, Daniel Picot and Patrick J. Loll of the Unversity of Chicago Department of Biochemistry and Molecular Biology. The three folding domains (36) are labeled: EGF-like domain, red; membrane binding domain, green; catalytic domain, purple. Glycosylation sites are shown in yellow and the heme group is shown in blue. The cyclooxygenase and peroxidase active sites are labeled by arrows.

function as a membrane binding domain. Interestingly, helices of this domain form the mouth of the cyclooxygenase active site channel. Three of the four helices are arranged with hydrophobic faces exposed to the exterior of the molecule, to form a hydrophobic surface patch for associating with membranes. The hydrophobic side chains are thought to enter the lumenal leaflet of the membrane bilayer in a monotopic orientation. It is possible that substrate arachidonate liberated from the membrane could directly enter the cyclooxygenase active site channel through the mouth of the membrane binding domain helices (36).

PGH synthase topology and localization

The topological model derived from the crystal structure is consitent with experiments that show PGH synthase is localized exclusively to the lumenal side of the ER membrane (89). Anti-PGH synthase antisera and selective membrane permeabilization were used to map the topology of PGH synthase domains in the ER membrane. Antisera generated against peptides encoding the N-terminus, C-terminus or middle of murine PGH synthase-1 were incubated with (a) plasma membrane permeabilized cells or (b) plasma membrane *and* ER membrane permeabilized cells. Staining of PGH synthase was observed only in cells that had permeabilized ER and plasma membranes. Thus the N-terminus, C-teminus and middle of PGH synthase are not accessible on cytoplasmic side of the ER membrane. In addition the three N-linked glycosylation sites are presumed to be in the lumen of the ER. Together these data demonstrate that PGH synthase lacks cytoplasmic domains and is localized exclusively to the lumenal side of the ER membrane. Figure 7 schematically represents this topological model.

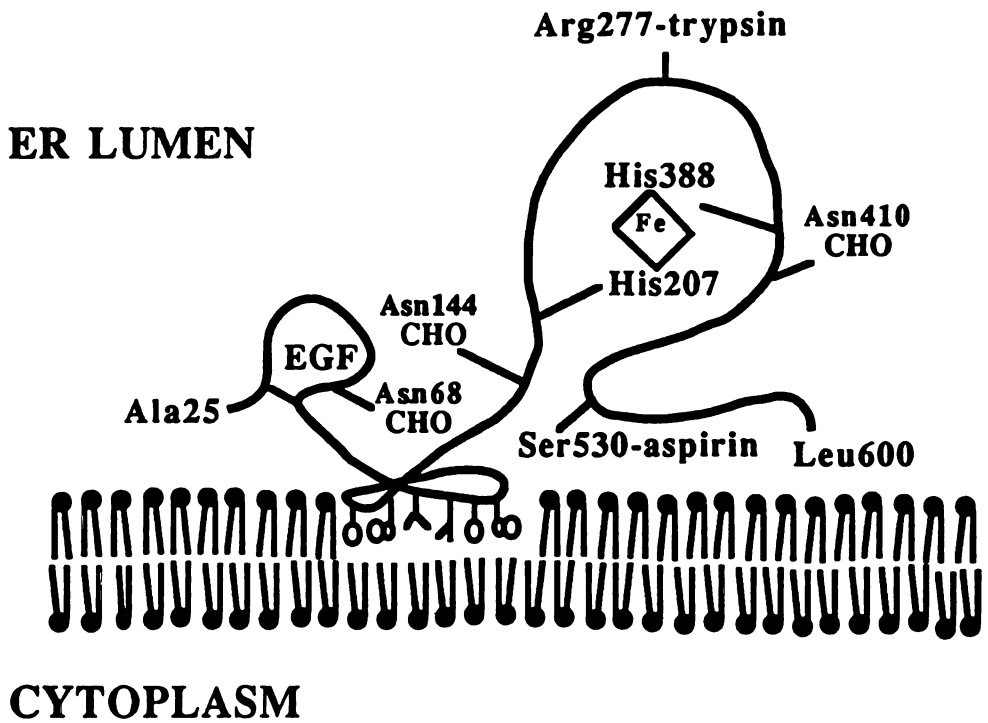

Figure 7. Model for PGH synthase membrane topology.

Subcellular localization of PGH synthase-1 in murine NIH 3T3 cells has been determined by indirect immunocytochemistry and immunohistochemical electron microscopy (90,91). PGH synthase-1 is localized to the ER and NE, but not the plasma membrane or mitochondrial membrane (91). PGH synthase-2 is also localized to the ER and nuclear membrane (92) . Although the two isozymes are localized to the same membranes, they differ in that PGH synthase-2 is concentrated in the NE, whereas PGH synthase-1 is uniformly distributed between the ER and NE (93).

PART 2: PROTEIN TARGETING

Part 2: Protein Targeting is divided in two sections. Section A: briefly covers targeting sequences which import proteins directly into their home organelle. These organelles include the nucleus, ER, and mitochondria. Section B: addresses sorting signals which are responsible for retention of proteins within the organelles of the secretory system, the ER, Golgi complex, lysosomes, and endosomes. Emphasis is placed on retention signals of the ER and NE which are the sites of localization of PGH synthases.

Introduction

In eukaryotes proteins are synthesized on ribosomes in the cytoplasm of the cell. Most proteins remain in the cytoplasm, but some are directed to other places within the cell in a phenomenon called protein sorting or protein targeting. Characterization of protein sorting domains is an area of general biochemical interest, because subcellular localization is often an important determinant of protein function.

The central feature of protein sorting is passage of proteins from the cytoplasm to a subcellular compartment by traversing the membrane system

which defines the compartment (94). 'Crossing' membranes can be accomplished in three ways: (a) Proteins may pass through pores in the membrane which act as gates between the cytoplasm and the interior of an organelle. This is the mode of entry into the nucleus. (b) Proteins may pass through the membrane directly by interacting with translocation machinery proteins in the membrane. This is the mode of entry of proteins targeted to the mitochondria and ER. (c) Proteins may enter a new organelle when membrane systems of different compartments come together and fuse. This is the mode of the secretory vesicle system. In each of these modes, sorting to the proper organelle is directed by elements of the protein itself, called sorting signals or targeting sequences. Sorting signals are usually encoded in the amino acid sequence of the protein, but in some cases oligosaccharide or fatty acid modifications of the protein are used .

Identification of targeting sequences of proteins usually involves two steps. First, the domain necessary for proper protein localization is identified. This is accomplished by analyzing a series of successive deletion mutants for changes in localization of the protein. The minimum deletion necessary to disrupt proper targeting identifies the putative targeting domain or sequence. Second, the putative targeting domain is appended to an unrelated protein, usually a cytosolic or secreted protein, which is endogenous to a different subcellular locale. If the appended targeting sequence is sufficient to re-target the unrelated protein to the new location, then the sequence demonstrates specific organelle sorting activity. Some sorting signals have been defined by specific consensus sequences. More often, however, sorting signal peptides for a particular organelle conform to a general pattern of characteristic length and biochemical character.

Section A:

Nuclear Targeting Sequences

Proteins targeted to the nucleus enter through the nuclear pore. This method of import is unique to the nucleus. The pore is a fixed channel, composed of soluble and integral membrane proteins, assembled to function as a gate between the cytoplasm and nuclear contents (95). Small molecules like water and ions diffuse passively through the pore, but proteins pass through the pore in an active, energy-requiring process. It is thought that proteins enter in a folded state and that the core aperture of the pore dilates to accommodate the size of the intact protein (96). Nuclear proteins are directed to the nucleus by a nuclear localization signal (NLS), a general motif which consists of a linear sequence of 4-8 amino acids rich in positively charged amino acids, lysine and arginine, and usually containing proline (97). Localization studies of the large T-antigen, a nuclear protein of the SV40 virus, were the first to define an NLS (98). In this case the targeting sequence is -PPKKKRKV-, a peptide found in the middle of the protein sequence. For some nuclear proteins the motif is split into two blocks of 2-4 amino acids each; this arrangement is called a bipartite nuclear targeting signal. The nuclear targeting signal can be located almost anywhere in the protein sequence; as expected, the signal is not cleaved but remains intact which is necessary, because nuclear proteins require resorting when the nucleus reassembles following mitosis.

ER and Mitochondrial Targeting Sequences (Signal Peptides)

Proteins enter the ER and the mitochondria in a process called translocation. The translocation process follows the same general steps in both organelles, but as expected the targeting sequences are distinct.

ER Targeting Sequences

Proteins destined for the ER and destinations further along the secretory pathway are directed to the ER by a sorting signal composed of hydrophobic amino acids, typically 10-30 residues in length (99,100). For example, the ER targeting sequences for murine PGH synthase-1 and -2 are $^{NH3+}$MSRRSLSLWFPLLLLLLLPPTPSVLL- and $^{NH3+}$MLFRAVLLCAALGLSQA- respectively. ER targeting sequences are at the N-terminus of the protein. When translocation is complete, the N-terminal targeting sequence is often removed by ER signal peptidase proteolysis; the resulting peptide encoding the targeting sequence is called the signal peptide, and the remaining portion is the mature protein. The term 'signal peptide' is best reserved for reference to a specific type of targeting sequence which is cleaved after targeting and does not remain in the mature protein. Signal peptides exert their targeting activity by specific association with the signal recognition particle (SRP) during translocation (101).

Proteins enter the ER by interaction with a poorly characterized translocation apparatus in the ER membrane (102). In the current model, a protein translocator protein forms a transient aqueous opening through which the extended, unfolded protein can cross (101). This is unlike import of intact proteins into the nucleus through a stationary pore with a fixed aqueous channel. Translocation into the ER is a multistep process simultaneous with protein translation. The general steps are outlined here. This topic is addressed in references 101-105. (a) Protein synthesis begins with free ribosomes translating the mRNA transcript. Translation progresses until the nascent N-terminal signal peptide is exposed. The signal-recognition particle (SRP) binds the signal peptide and ribosome; translation pauses momentarily. (b) The cytosolic complex moves to the ER when the SRP binds

the SRP receptor in the ER membrane; concurrently the ribosome is bound by the ribosome receptor, another integral ER membrane protein. (c) Protein translation resumes, and the nascent protein enters the ER lumen cotranslationally. Translocation is thought to occur via the protein translocator which creates an aqueous pore for import. (d) When translation is complete, the signal peptide is cleaved, and the mature protein is released in the ER lumen.

Soluble proteins are released into the lumen of the ER after translocation, but integral membrane proteins remain associated with the ER membrane. Translocation of integral membrane proteins with single or multiple transmembrane domains is a function of multiple targeting sequences called start-transfer and stop-transfer and signal anchor sequences. This topic is addressed in references 106 and 107.

Mitochondrial Targeting Sequences

Mitochondrial proteins are imported to the mitochondria matrix by a translocation process similar to ER translocation. Mitochondrial translocation occurs post-translationally; proteins must be unfolded prior to translocation. Unfolding outside the mitochondria and refolding of the translocated protein in the matrix depends on mitochondrial hsp70 chaperonin, and ATP (108).

Components of the translocation machinery are not well characterized. A receptor in the mitochondrial membrane binds the mitochondrial signal peptide and facilitates translocation (109). The signal peptide is an N-terminal sequence composed of 20-80 residues with periodic spacing of positively charged residues. For example, the mitochondrial sorting signal of subunit IV of cytochrome oxidase is $^{NH3+}$MLSLRGSIRPPKPATRTL- . It is thought that

mitochondrial signal peptides form an amphipathic α-helix with a positively charged face. This interacts with a mitochondrial membrane translocation receptor likely by interaction at the positive face of the signal peptide α-helix. After translocation the targeting sequence is cleaved. Targeting proteins to mitochondrial subcompartments and internal membranes within the organelle requires additional sorting signals.

Section B: Retention Sequences in the Secretory Pathway

The secretory pathway involves the movement of proteins through a series of membrane compartments interconnected by vesicle budding and fusion. Proteins carried to the plasma membrane in transport vesicles encounter these compartments sequentially: the ER, intermediate compartment, cis Golgi, Golgi stack, and trans Golgi. The movement of proteins in the secretory pathway is nonselective; it occurs in forward bulk flow fashion by default (110,111). Therefore, once a protein is translocated to the ER lumen it assumes a fate for eventual secretion. However, each of these compartments contain a characteristic set of lumenal contents and integral membrane proteins which resist the forward flow and remain as resident proteins. These proteins carry signals that allow them to be retained or retrieved and returned to their home compartment. In some cases the vesicular journey of a retained or retrieved protein can be ascertained by analysis of its oligosaccharide modifications. This is true because the ER and various regions of the Golgi differ in their content of oligosaccharide modifying enzymes. Thus, the route through these compartments can be monitored by the sequential changes that occur to their oligosaccharide side chains of glycoproteins (86).

Three retention signals have been well characterized in the secretory pathway. The -KDEL and -KKXX signals function to retrieve and retain proteins in the ER (112). The mannose-6-phosphate modification directs acid hydrolases to lysosomes (113). It is interesting that the retention sequences are encoded by a strict consensus sequence, unlike the targeting signals for import into the ER, mitochondria and nucleus.

It is important to note that the majority of proteins in the endocytic and secretory pathways are carried as cargo proteins, soluble components of the vesicles. They are targeted not by their own sequences, but indirectly by the targeting mechanisms which specifically direct vesicular traffic. Vesicular targeting and trafficking, how a vesicle originates and determines its proper destination among the many membranous cellular compartments, is a very complex process that involves cytosolic proteins and integral membrane proteins of the budding and fusing vesicular membranes. Recent reviews in this field are found in references 114-117.

ER Retention Signal—KDEL

Resident proteins in the ER may be soluble, membrane associated or integral membrane proteins. Integral membrane proteins are of two types based on the orientations of their C-termini. The C-termini of type I integral membrane proteins are found in the cytoplasm; in type II proteins, the C-temini are in the lumen of the ER. Proteins with multiple membrane spanning domains are designated as polytopic; those with a single span, bitopic (118).

Two ER retention sequences have been identified for resident ER proteins. The -KDEL retention sequence retains soluble proteins and type II integral membrane proteins (119). The -KKXX retention sequence retains type I

integral membrane proteins (120). While these two ER retention sequences have been identified as retention mechanisms for some ER proteins, there are many more which do not possess these particular sequences. For example, it is not known how resident integral membrane proteins of the ER avoid localization to those regions of the ER membrane which are used for the formation of secretory/transport vesicles. Obviously, additional mechanisms are at work to maintain the full complement of ER occupants.

Soluble and type II membrane proteins can be retained in the ER by a C-terminal tetrapeptide sequence (119,121,122). The consensus sequence is -KDEL in mammals (123) and plants (124), and -HDEL in yeast (114). There are, however, a number of functional ER retention signals which use other residues at the first and second position of the tetrapeptide (119). The last two residues, glutamate and leucine, are nearly invariant (119); even a conservative change of leucine to valine (124,125) or isoleucine (124) disrupts retention activity. (It should be noted that a single report does show retention activity by -QEDL (126).) Interestingly, the retention sequence is not dependent on charge conservation of the amino acid side chains (127,128). The notation '-XXEL,' is possibly an over simplification of the consensus sequence requirement, but better represents the variety of eligible residues for the first and second position. Most studies show that the tetrapeptide itself is fully competent for retention activity, but several reports suggest that upstream residues may influence the efficiency of retention (124,125). Overall, it appears that the -KDEL retention machinery exhibits high specificity for the third and fourth positions of the tetrapeptide.

Investigation of this retention sequence began when a common mammalian C-terminal sequence, -KDEL, was identified in several soluble ER resident proteins (129,130). Pelham and colleagues tested the retention

hypothesis in studies (123) using two proteins: immunoglobulin binding protein (BiP), an ER resident protein, and lysozyme, a secreted protein. When -KDEL was deleted from BiP, the protein was not retained in the ER, but was secreted; when -KDEL was appended to lysozyme, the protein was not secreted but was retained in the ER. It has also been shown that -KDEL is functional for type II integral membrane proteins (122,131). When -KDEL is appended to a type II integral plasma membrane protein, the protein is retained in the ER membrane and no longer localized in the plasma membrane (131).

The mechanism of ER retention involves interaction of the -KDEL retention sequence and the -KDEL receptor. The receptor does not 'tether' the ER resident protein to the ER membrane (132). In these experiments the diffusion rates in the ER for native BiP, a BiP mutant lacking -KDEL, and a secreted protein were shown to be similar. This suggests BiP is not immobilized by a receptor interaction. Instead, it is thought that the -KDEL receptor retrieves ER resident proteins from a post-ER compartment of the secretory pathway. This receptor retrieval or protein recycling model is strongly supported by ER resident proteins which exhibit glycosylation modifications catalyzed by enzymes localized exclusively in post-ER compartments. For example, a lysosomal enzyme appended with the -KDEL signal accumulates in the ER as expected, but is still modified by GlcNAc-phosphotransferase, which is thought to be a cis Golgi enzyme (133); a secreted protein in yeast appended with the -HDEL signal is retained in the ER, but possesses α1-6 mannose linkages which are processed in the early Golgi (134). It is thought that retrieval occurs at the cis Golgi or at the salvage compartment, a collection of smooth vesicles which lie between the transitional elements of the ER and the cis Golgi.

A -KDEL receptor has been cloned and identified as a 23-26 kDa integral membrane protein (135-138). Hydropathy plot analysis predicts a structure with seven transmembrane domains; the C-terminus is thought to be exposed to the cytoplasm (139). (It should be noted this is unlike G-protein linked receptors of signal transduction pathways which also have seven transmembrane domains but a *lumenal* C-terminus.) Functional domains of the receptor have been determined by mutational analysis (139). The transmembrane domains are involved in ligand binding activity and ligand specificity, and a single aspartate residue in transmembrane domain VII is responsible for receptor recycling activity. The domain responsible for salvage compartment localization has not yet been determined.

Consistent with a model of receptor recycling, not fixed receptor retention, it is supposed that ligand binding is controlled by a lumenal environmental factor unique to the salvage compartment. In the ER the receptor and ligand are not associated, but in the post-ER compartment 'escaped' ligands do bind to the receptor. Compartment acidity was suggested as a possible regulator of binding, when it was determined that affinity for the ligand is greatly increased under acidic conditions (pH 5.0) (140). More study is required to determine if this is a valid hypothesis for *in vivo* conditions. When the tetrapeptide sorting signal binds the -KDEL receptor, it is thought that a conformational change exposes the aspartate responsible for recycling in such a way to initiate entry of the receptor into the retrograde transport pathway.

ER Retention Sequence—KKXX

A different mechanism of retention has been identified for type I membrane proteins (those with a cytoplasmic C-terminus) that are localized in the ER. The dilysine motifi, -KKXX or -KXKXX, located at the extreme C-

terminus of some integral type I ER membrane proteins has been shown to retain proteins in the ER (120,141-143). Carbohydrate modifications of these proteins suggest that these ER residents are retrieved from a post-ER compartment (141,143), similar to the path taken by proteins of the -KDEL retention system. Proteins of the putative retrieval machinery are not yet identified, but it is hypothesized that a cytoplasmic -KKXX binding protein interacts with -KKXX C-terminal sequences in the post-ER compartment and directs them to the ER via the the retrograde transport pathway (141,144). One report suggests that retrieval of -KKXX terminating proteins may be initiated from multiple positions along the exocytotic pathway (145).

Localization in the NE

The targeting sequence of proteins imported directly into the nucleoplasm was described in **Part 2: Section A**. Another important group of nuclear proteins are those in the NE. The NE is composed of three distinct membrane domains: the outer membrane which is continuous with the ER, the nuclear pore membrane, and the inner membrane (146). The lumen formed by the inner and outer membranes is the perinuclear space. The membrane domains can be distinguished from one another by the different proteins found in each. How are these proteins sorted within the membrane? It is easy to imagine that proteins in the outer membrane could arrive there simply by localized protein translocation coincident to that specific area of the ER. Other questions arise upon further consideration. It is not known how these proteins are held resident in this specific region of the ER membrane (the outer nuclear membrane), how proteins physically move from the ER to the outer and then to the inner nuclear membranes, how inner and outer membrane proteins are distinguished, or how these proteins reassemble to

the proper nuclear/cytoplasmic face after mitosis. Based on current models of protein synthesis and targeting, it is assumed that targeting sequences direct proteins to the proper domains after translocation into the ER. It is also assumed that inner membrane proteins are distributed there by moving in the lateral plane of the membrane, diffusing through the outer and pore membrane domains.

Localization to the nuclear pore membrane

The targeting sequence for one protein member of the nuclear pore complex, gp210, has been identified (147). The protein is a bitopic, type I integral membrane protein with the majority of its mass located in the N-terminal, lumenal domain. These studies show that the residues of the transmembrane domain are sufficient for targeting. It is thought that the targeting sequence -SYQVMFFTFFALLAGTAVTIIAY- forms an α-helix in the membrane and is targeted to the pore membrane domain by specific protein-protein interactions with other integral pore membrane proteins (147).

Localization to the inner nuclear membrane

The targeting sequence which directs proteins to the inner nuclear envelope is not known. The lamin B receptor (LBR) is a type I integral protein of the inner nuclear membrane, that is composed of a 208 amino acid nucleoplasmic domain and eight transmembrane domains. The nucleoplasmic domain binds lamin B and DNA (148). It has been postulated that the LBR is localized or retained in the inner nuclear membrane by its interaction with lamin B (148,149). It also seems plausible by inference from gp210 that targeting sequences could be in the transmembrane domains (147). Further work is required to identify the retention sequence.

Dissertation Overview

The research presented in this dissertation focuses on aspects of PGH synthase localization. The impetus for this work has been a desire to understand how PGH synthase isozymes are localized in the cell and how their differential localization might influence isozyme-specific biological activities. Chapter 2 presents an investigation of a putative ER retention signal in PGH synthases. Chapter 3 addresses the intracellular localization of PGH synthase-2. Chapter 4 presents preliminary work to determine the role of the unique 18 amino acid sequence in PGH synthase-2 localization. Chapter 5 investigates the subcellular localization of cPLA, an enzyme which provides unesterified archidonate for PGH synthase substrate.

CHAPTER 2

LOCALIZATION OF PROSTAGLANDIN ENDOPEROXIDE SYNTHASE-1 TO THE ENDOPLASMIC RETICULUM AND NUCLEAR ENVELOPE IS INDEPENDENT OF ITS C-TERMINAL TETRAPEPTIDE, -PTEL

Abstract

Both prostaglandin endoperoxide H (PGH) synthases-1 and -2 are membrane-associated proteins localized to the endoplasmic reticulum (ER) and NE. The carboxyl terminal tetrapeptides of PGH synthases -1 and -2 are of the form -P/STEL. These sequences are similar to the -KDEL retention signal sequence characteristic of many proteins localized to the ER. To determine if the -PTEL sequence (residues 597-600) functions as an ER retention signal for ovine PGH synthase-1, we prepared and analyzed five mutants (L600N, L600R, L600V, E599Q and Δ597), all having modifications that would be expected to alter the subcellular location of PGH synthase-1 if the -PTEL sequence were involved in ER targeting. Native ovine PGH synthase-1 and each of the five mutants were subcloned into the pSVT7 expression vector and were expressed transiently in *cos*-1 cells. The L600N, L600R, E599Q, and Δ597 mutants retained both cyclooxygenase and peroxidase activities. Moreover, when subjected to immunocytofluorescent staining, *cos*-1 cells expressing native and mutant enzymes showed similar patterns of fluorescence corresponding to ER and NE localization. Finally, culture media bathing *cos*-1 cells transfected with native or mutant PGH synthases were tested for secreted PGH synthase-1 protein by Western blotting, but no PGH

synthase-1 was detected in any of the culture media. Our results demonstrate that mutations in the C-terminal sequence -PTEL do not change the subcellular location of ovine PGH synthase-1. Thus, targeting of PGH synthase-1 to the ER can occur independent of its -PTEL sequence.

Introduction

Both isozymes of prostaglandin endoperoxide H (PGH) synthase catalyze the conversion of arachidonic acid to prostaglandin endoperoxide H_2 (PGH_2) in the first step of prostanoid biosynthesis. PGH synthases-1 and -2 are integral ER membrane proteins, in that they can only be solubilized by detergent extraction (52). Data from immunocytofluorescence (90,92) and immunoelectron microscopy (91) have demonstrated that PGH synthases are present in the ER and NE, with PGH synthase-2 being more highly concentrated in the NE (93). Neither isozyme appears to be present in the plasma membrane. The mechanisms involved in the organelle targeting of these proteins have not been determined.

Proteins, other than those which occur in the cytosol, are initially translocated to the ER, where targeting signals encoded in the amino acid sequence direct further transport. Proteins lacking targeting sequences are thought to follow a default pathway leading either to secretion for soluble proteins or transport to the plasma membrane, in cases of membrane proteins (121). The ER itself contains many proteins which resist bulk flow secretion. Localization of some resident ER proteins is dependent on a C-terminal tetrapeptide retention signal, -Lys-Asp-Glu-Leu (-KDEL) (119). While the amino acid sequence of this retention signal can vary somewhat, depending on the species and the protein, the terminal glutamate and leucine residues are essential (119). Functional ER retention signals have been shown for both soluble (119,128) and integral membrane proteins (122) (Figure 8). Secreted proteins appended with the C-terminal tetrapeptide are no longer exported, but remain in the ER (123). Furthermore, integral proteins of the plasma membrane can be converted to integral ER membrane proteins by addition of a C-terminal -KDEL sequence (131). In addition the retention

RESIDENT ER PROTEINS	BiP (rat)	K D E L
	BiP (*P. falciparium*)	S D E L
	Liver esterase	H T E L
	Sec20p	H D E L
PGH SYNTHASES	Sheep-1	P T E L
	Human-1	S T E L
	Rat-1	S T E L
	Mouse-1	S T E L
	Mouse-2	S T E L
	Rat-2	S T E L
	Human-2	S T E L
	Chicken-2	S A E L

Figure 8. Comparison of C-terminal amino acid sequences of PGH synthases -1 and -2 and several resident ER proteins. Amino acid sequences of ER retention signals are shown for several resident ER proteins: rat BiP (123), *P. falciparium* BiP (150), mouse liver esterase (128), and Sec20p from yeast (122). Carboxy-terminal amino acids for PGH synthase isozymes are aligned for sequence comparison: sheep PGH synthase-1 (54-56), mouse PGH synthases -1 (45) and -2 (58), human PGH synthases -1 (60) and -2 (61,62), rat PGH synthases -1 (63) and -2 (63,64), and chicken PGH synthase-2 (57).

mechanism appears to be somewhat general as the ER localization of resident ER proteins is preserved even when the protein is transiently expressed in a different species (123,128).

Ovine (54-56), murine (45,58), human (60-62), and rat (63,64) PGH synthases -1 and -2 all have C-terminal sequences (-P/STEL) which closely resemble the -KDEL retention signal (Figure 8). The experiments described in this report were designed to determine if the targeting of ovine PGH synthase-1 to the ER requires its C-terminal -PTEL sequence.

Materials and Methods

Materials.

Oligonucleotides used as primers for mutagenesis were prepared by the Michigan State University Macromolecular Structure and Sequencing Facility. *Sal* I and T4 DNA ligase were obtained from Boehringer Mannheim. Endoglycosidase H_f was obtained from New England BioLabs. Mutagenesis reagents, a Muta-Gene kit, Affi-Gel 10, and anti-rabbit IgG horseradish peroxidase conjugate were obtained from BioRad. Dideoxy sequencing was performed according to the method of Sanger (151) using a Sequenase kit obtained from United States Biochemical Corp. CsCl was from Var Lac Oid Chemical Company. Dulbecco's modified Eagle medium (DMEM) was obtained from GIBCO. Bovine calf serum and fetal calf serum were obtained from Hyclone. Tissue culture plates and glass coverslips were from Corning. Arachidonic acid was obtained from Cayman Chemicals. BA85 (0.45 μm) nylon-supported nitrocellulose was from Schleicher & Schuell. An ECL (Enhanced Chemiluminescence) kit was from Amersham. FITC-labeled goat anti-rabbit IgG (whole molecule) was from Sigma. Slowfade bleaching retardant was obtained from Molecular Probes. X-OMAT AR X-ray film was from Kodak. All other reagents were from standard sources.

Site-Directed Mutagenesis.

Ovine PGH synthase-1 cDNA was subcloned into the *Sal* I restriction site of M13mp19 for site-directed mutagenesis. Oligonucleotides of 24 bases were synthesized to encode mutations at the C-terminus of ovine PGH synthase-1 as shown (superscripts indicate oligonucleotide numbering described previously (ovine cDNA paper)):

L600R 5′-^{1965}CCACCCACAGAGAGATGAAGGGGCT^{1989}-3′

L600V 5′-^{1965}CCACCCACAGAGGTATGAAGGGGCT^{1989}-3′

L600N 5′-^{1965}CCACCCACAGAGAACTGAAGGGGCT^{1989}-3′

E599Q 5′-^{1965}CCACCCACACAACTCTGAAGGGGCT^{1989}-3′

Δ597 5′-^{1954}GGGTGGAGCGGCCATAGACAGAGCTCTG^{1981}-3′

Mutagenesis was performed using a Muta-Gene kit patterned after the method of Kunkel (152). Native and mutant ovine PGH synthase-1 cDNAs were subcloned into the *Sal* I restriction site of pSVT7 expression plasmid (153) and sequenced to verify mutations (151).

Cell Culture, Transient Transfection, and Microsome Preparation.

Cos-1 cells (ATCC CRL 1650) were cultured in 100 x 20 mm tissue culture plates using DMEM supplemented with 8% calf serum, 2% fetal calf serum in a 7% CO_2, water-saturated atmosphere at 37° C. Expression plasmids were transfected into *cos*-1 cell cultures grown to 60-80% confluency using the DEAE dextran and chloroquine method (154). About 40 hours after transfection, cells were harvested and processed as previously described (45). Briefly, transfected cells were harvested in 0.1 M sodium phosphate, 0.9% NaCl, pH 7.4 (PBS) using a rubber scraper and collected by centrifugation for 5 minutes at 3000 x g. Cell pellets were resuspended in 0.1 M Tris HCl, pH 8.0 at 4° C, sonicated using a microtip attachment, and centrifuged at 4° C for 10 minutes at 10,000 x g to remove cellular debris and nuclei. The resulting supernatants were centrifuged at 4° C for 1 hour at 100,000 x g. The resulting pellet of microsomal membranes was resuspended using a 2 ml Dounce homogenizer in 0.1 M Tris HCl, pH 8.0, at a concentration of 0.05-0.1 ml/plate

of transfected cells. Freshly prepared microsomes were used for Western blot analyses and enzyme assays.

Western Blot Analyses and Enzyme Assays.

Proteins were separated and visualized using standard Western blot procedures. Sample loading buffer was added to samples, and protein gels (10 μg protein/lane) were electrophoresed at 40 mA for about 2.5 hours (155). Transfer to nylon-supported nitrocellulose was performed using a BioRad semi-dry transfer apparatus with 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3 transfer buffer at 100 mA (10 V) for 1 hour, according to the method of Towbin *et al.* (156). Detection of PGH synthase protein was performed essentially as described in the directions of the ECL kit. Protein blots were blocked at 4° C for 4-14 hours in 3% nonfat milk, 0.05% Tween 20 in 25 mM Tris HCl, 2.7 mM KCl, 0.14 M NaCl, pH 7.3 (TBS). Affinity-purified antibodies generated from peptides encoding either the N-terminal residues A24-C35 or the C-terminal residues P583-E594 of ovine PGH synthase-1 (89) were diluted 1:1000 in 1% nonfat milk, 0.05% Tween 20 in TBS, and incubated with the blot for 1-3 hours on a platform rocker. Goat anti-rabbit IgG horseradish peroxidase conjugate was diluted 1:2000 in 1% nonfat milk, 0.05% Tween 20 in TBS, and incubated with the blot for 1 hour. Visualization of protein was performed using ECL kit reagents. X-ray film was exposed for 5-30 seconds and then processed.

Cyclooxygenase activity was measured using an oxygen electrode assay (83), and peroxidase activity was monitored spectrophotometrically as previously described (157).

Immunofluorescence Staining.

Fluorescence staining to determine the subcellular location of PGH synthase-1 was performed essentially as previously described (92). Briefly, *cos*-1 cells were cultured on glass coverslips and transfected as described above. Cells were usually processed 40 hours following transfection, but in some cases, at 24, 30, 38 or 41 hours post-transfection. Intracellular staining was performed with formaldehyde-fixed, saponin-permeabilized (0.2%) cells. Cells were incubated for 1 hour with affinity-purified, peptide-specific antibodies generated against the N-terminus of ovine PGH synthase-1 (A25 to C34) (89) diluted 1:20 into 0.2% saponin, 10% calf serum in PBS. After washing, coverslips were incubated for 1 hour with a 1:40 dilution of FITC-labeled goat anti-rabbit IgG (whole-molecule). Coverslips were rinsed and mounted in Slowfade bleaching retardant. Staining for cell-surface antigens was performed at 4° C with unfixed, nonpermeabilized cells which were incubated with primary and secondary antibodies as described above, but omitting saponin. After incubations with the antibodies, the cells were fixed with formaldehyde and mounted in Slowfade beaching retardant. Photomicroscopy was performed at 400X magnification with 2-minute exposures using Kodak Tri-X Pan 400 film and a Leitz Orthoplan fluorescence microscope.

Immunoaffinity Chromatography.

Immunoaffinity columns were prepared using a monoclonal antibody, cyo-7, generated against sheep seminal vesicle PGH synthase-1 (158). Columns were prepared as follows (54): cyo-7 (1 mg/ml) was dialyzed against 0.1 M Hepes pH 7.5 and then incubated at 4° C for 12 hours with 10 ml of Affi-Gel 10. Unreacted sites were blocked by treatment with 10 ml of 0.1 M

ethanolamine. Two ml aliquots of antibody-coupled matrix were loaded into 5 ml syringes and then washed extensively with 0.1 M Tris HCl, pH 8.0, containing 0.2% Tween. Cell culture media from *cos*-1 cells transfected with native PGH synthase-1 or the L600R or Δ597 mutant enzymes were collected and centrifuged at 4° C for 5 minutes at 5,000 x g to precipitate cellular debris. After filtration through a 0.22 μm filter, the supernatants were percolated twice over individual cyo-7-Affi-Gel 10 columns. Columns were washed with 0.1 M Tris HCl, pH 7.5, containing 0.02% Tween 20 and then eluted with 2 ml of acetic acid. The eluate was immediately neutralized with 250 μl of 2 M Tris (total sample volume 2.25 ml). Microsomes from the same transfected cells from which media were removed were prepared as described above and homogenized in 2.25 ml of 0.1 M Tris HCl, pH 7.5. Sample loading buffer was added to the column eluates and microsomal samples, and SDS PAGE and Western blot analysis were performed as described above.

Endoglycosidase H Digestion.

Endoglycosidase H digestion was performed as previously described (47). Microsomes (2 mg/ml) were solubilized in 0.5% SDS at 100°C for 2 minutes and then stored on ice for 5 minutes. Solubilized microsomes were digested for 12 hours at 37° C with 5,000 units of endoglycosidase H_f in 50 mM sodium citrate, pH 5.5. Samples were subjected to SDS PAGE using 10 μg of protein per lane.

Results

Mutations which disrupt functional ER retention signals in other proteins (7,9,10,32,33) were generated in the ovine PGH synthase-1 cDNA. The C-terminal leucine residue (L600) was mutated to an asparagine (L600N), arginine (L600R), or valine (L600V), and the penultimate glutamate was mutated to a glutamine (E599Q); in addition, a truncation mutant (Δ597) was generated by changing proline 597 to a stop codon. These mutant PGH synthase-1 cDNAs were subcloned into the expression plasmid pSVT7 and expressed in *cos*-1 cells. To determine the effect of the mutations on the structural and catalytic properties of the enzyme, we first performed Western blot analyses and measured enzyme activities (Figure 9). Western blot analyses of *cos*-1 microsomal protein preparations showed that the PGH synthase-1 mutants, except for the L600V mutant, are expressed and have the same electrophoretic mobilities as native ovine PGH synthase-1 (Mr = 72,000). The mutant enzymes, again except for the L600V mutant, were catalytically active. Cyclooxygenase activities ranged from 16% to 25% of native PGH synthase-1 enzyme activity; similarly, peroxidase activities ranged from 9% to 16% of native enzyme activity. We conclude that the C-terminal mutations L600N, L600R, E599Q and Δ597 are permissible with relatively minor changes in the conformation of ovine PGH synthase-1. Additional experiments performed on the L600V mutant are described below.

Immunocytochemistry was performed to determine if altering the C-terminal tetrapeptide, -PTEL, affected the subcellular location of ovine PGH synthase-1. Intracellular staining of *cos*-1 cells transfected with cDNAs encoding native or mutant L600N, L600R, L600V, E599Q, and Δ597 PGH synthases -1 was performed with either detergent-permeabilized or non-permeabilized cells. No cell surface staining was observed with non-

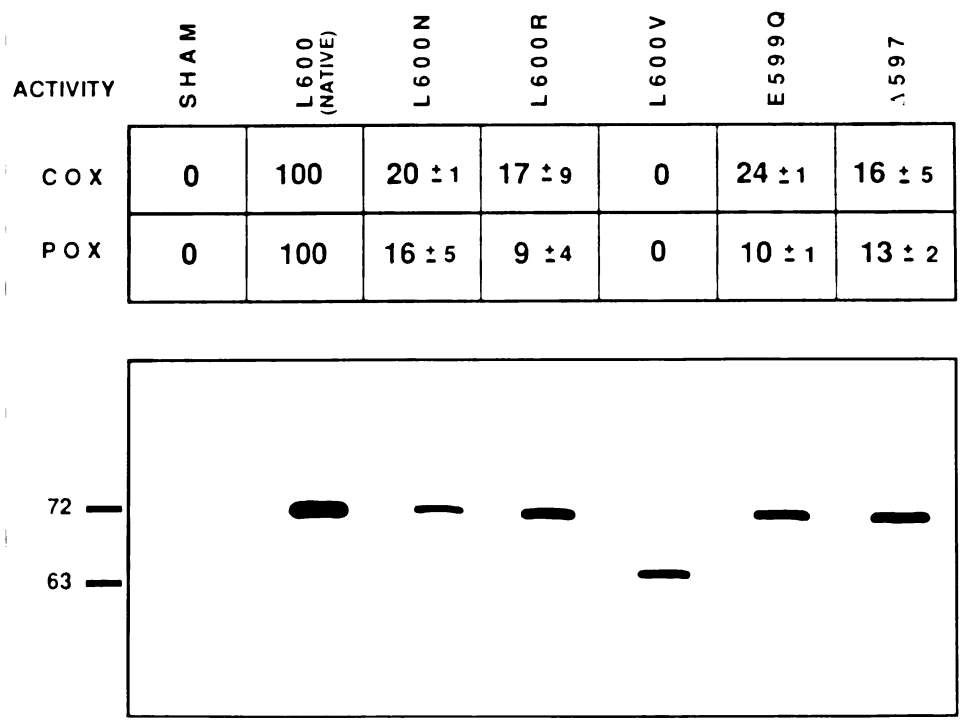

Figure 9. Enzyme activity and expression of ovine PGH synthases-1 having carboxyl terminal mutations. Native and mutant ovine PGH synthases-1 were expressed in *cos*-1 cells, and microsomes were prepared for assays of cyclooxygenase and peroxidase activities (upper panel) and SDS PAGE/Western blotting (lower panel) as described in the text. Cyclooxygenase (COX) and peroxidase (POX) activities are expressed as a percentage of native enzyme activities. Cyclooxygenase activities were determined from two to seven separate transfections; peroxidase activities from one to three separate transfections. In different experiments, cyclooxygenase and peroxidase activities of native PGH synthase ranged from 213 to 269 nmol 20:4/min/mg and 274 to 344 nmol H_2O_2/min/mg, respecitvely. The anti-peptide antibodies used for Western blotting were directed against the N-terminus of ovine PGH synthase-1 (89). Data are expressed +/- SEM.

permeabilized *cos*-1 cells transfected with native PGH synthase-1 or with any of the PGH synthase mutants (Figure 10). In permeabilized cells, intracellular staining patterns characteristic of the ER and NE were observed for both native and mutant PGH synthases (Figure 10). Sham-transfected *cos*-1 cells showed no detectable PGH synthase-1 (data not shown). The results presented in Figure 10 are for cells stained approximately 40 hours post-transfection. Overexpression of PGH synthase by transient transfection can significantly disrupt the morphology of *cos*-1 cells and result in areas of very intense immunofluorescence. To determine if subtle changes in localization were discernible with *cos*-1 cells exhibiting less intense staining and a more characteristic morphology, immunocytochemistry was also performed at earlier times (24-30 hours) following transfections. However, the immunofluorescence staining patterns observed at both earlier and later time points were the same, demonstrating the same ER and NE localization (data not shown) for native ovine PGH synthase-1 and the Δ597 PGH synthase-1 mutant.

No cell surface staining was observed with any of the C-terminal PGH synthase-1 mutants, although the epitope (A25-C35) would be expected to be on the outside of the cell were any of the mutants not retained in the ER (89). However, if PGH synthase-1 were relatively loosely associated with membranes, mutation of a functional retention signal would result in secretion of the protein. To determine if C-terminal mutations caused secretion of any of the PGH synthase-1 mutants, the media and microsomes from *cos*-1 cells expressing native PGH synthase-1 and the L600R and Δ597 mutant PGH synthases -1 were assayed for PGH synthase-1 protein by Western blot analysis. Although native PGH synthase-1 and the L600R

Figure 10. Subcellular localization of native and mutant ovine PGH synthases-1. *Cos*-1 cells were transfected with ovine PGH synthase-1 or the E599Q or Δ597 mutant PGH synthases -1. The subcellular locations of the native and mutant PGH synthases were determined by indirect immunofluorescence using purified peptide-specific rabbit antibodies generated against the N-terminus of ovine PGH synthase-1 (89) and FITC-conjugated goat anti-rabbit IgG. Staining of the ER and other internal membranes was performed using permeabilized cells (treated with saponin); staining of the cell surface was performed using non-permeabilized cells. Photomicroscopy was performed on a Leitz Orthoplan fluorescence microscope at 400X magnification with 2-minute exposures using Tri-X Pan 400 film.

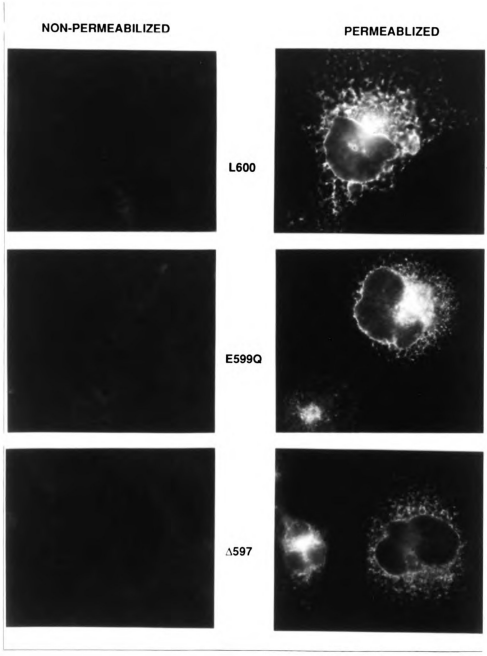

FIGURE 10

and Δ597 mutant PGH synthases -1 were present in microsomes, they were not detectable in culture media (Figure 11). Thus, mutation of the putative ER retention signal does not result in secretion of mutant PGH synthase-1. These data, along with the results of immunocytochemistry, lead us to conclude that C-terminal mutations of the -PTEL sequence do not cause a change in the subcellular location of PGH synthase.

The physico-chemical properties of the L600V PGH synthase-1 mutant differed from those obtained with our other mutants. No cyclooxygenase or peroxidase activity was detected with the L600V mutant. Subcloning and sequencing of a second L600V PGH synthase-1 mutant cDNA was repeated, and the new construct was transfected in *cos*-1 cells. Again, no enzyme activities were detected with microsomes prepared from cells transfected with this second L600V mutant construct. Furthermore, Western blot analysis showed that the L600V PGH synthase-1 protein migrates with an Mr = 63,000, an apparent decrease of 9 kDa compared with native PGH synthase-1 (Mr = 72,000; Figure 9). Initially, we suspected that the abnormal size of the L600V PGH synthase-1 was caused by proteolytic degradation of misfolded protein. However, when peptide-directed antibodies specific for either the N- or C-termini of ovine PGH synthase-1 (89) were used in Western blot analysis of L600V PGH synthase microsomes, each antibody reacted with the 63 kDa L600V protein (data not shown). Thus, the smaller size of the L600V mutant is not attributable to proteolytic degradation from either of the protein termini. Furthermore, the decrease in the apparent molecular weight of L600V PGH synthase-1 was not caused by improper N-glycosylation. Endoglycosidase H treatment of microsomes prepared from *cos*-1 cells expressing either the native or the L600V mutant PGH synthase-1 caused a decrease of 6 kDa for each protein (Figure 12). As discussed below, we suspect

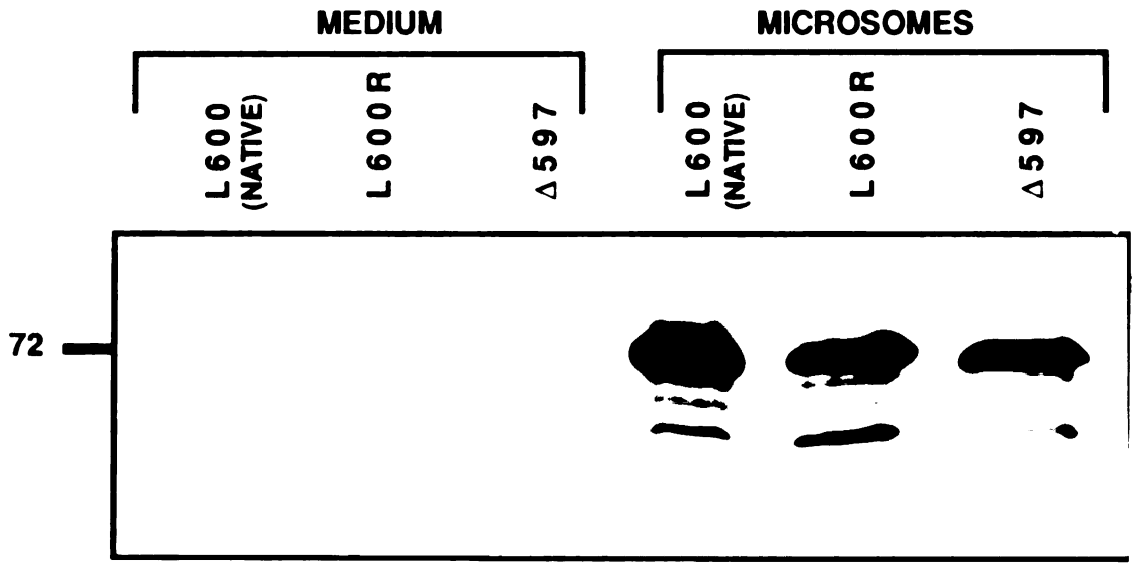

Figure 11. Western blotting of PGH synthase-1 in microsomes and media from cos-1 cells transiently expressing native ovine PGH synthase-1, L600R PGH synthase-1, or Δ597 PGH synthase-1. Native ovine PGH synthase-1 or the L600R or Δ597 mutant PGH synthases -1 were expressed in *cos*-1 cells. Microsomes were isolated from the transfected cells; in addition, the media bathing the cells were subjected to immunoaffinity column chromatography as described in the text. Microsomal preparations and samples of column eluates were prepared in equal volumes and 50 µl of each were subjected to Western Blot analysis.

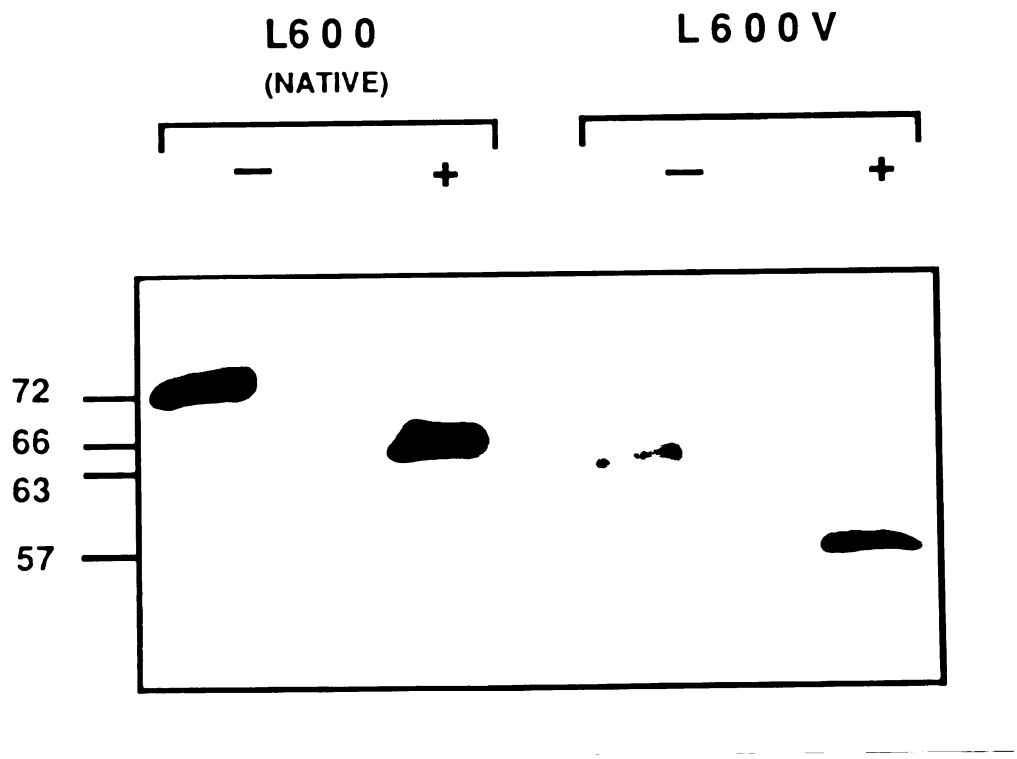

Figure 12. Endoglycosidase H treatment of native PGH synthase-1 and L600V mutant PGH synthase-1. Ovine PGH synthase-1 and the L600V mutant PGH synthase-1 were each expressed by transient transfection of *cos*-1 cells, and microsomes were prepared and treated with (+) or without (-) endoglycosidase H and subjected to Western blot analyses as described in the text.

that expression of the cDNA encoding the L600V mutant in the pSVT7 expression vector must lead to aberrant mRNA splicing.

Discussion

Earlier studies have indicated that PGH synthases -1 and -2 are membrane-associated proteins found in the ER and the NE (89,91,92). Our experiments were designed to test the hypothesis that the C-terminal tetrapeptide, -PTEL, of ovine PGH synthase-1 is required to target the enzyme to the ER. This hypothesis was based on the sequence similarities between the C-terminal tetrapeptides of PGH synthases and the -KDEL ER retention signals of other luminal ER proteins (Figure 8). Functional ER retention signals of a number of resident ER proteins consist of at least four residues at the C-terminus with invariant penultimate glutamate (E) and C-terminal leucine (L) residues (119). The other two residues vary, depending on the species and the protein. For example, -KDEL and -SDEL are ER retention signals for immunoglobulin heavy chain binding protein (BiP) from rat (123) and *P. falciparium* (150), respectively; -HTEL is the ER retention sequence for rabbit liver esterase (128), a soluble protein in the ER lumen; and -HDEL is a functional ER retention sequence for Sec20p (122), a type II integral ER membrane protein from *S. cerevisiae.* A C-terminal -XXEL sequence is found in all PGH synthases -1 and -2 sequenced to date (Figure 8). This sequence conservation suggests that these residues play a role in the function of PGH synthases.

Proteins translocated into the lumen of the ER that lack signals for subcellular organelle targeting are transported to the plasma membrane by vesicles in the secretory pathway; membrane-associated proteins are retained within the plasma membrane, while soluble proteins are released from the cell when the vesicular membrane fuses with the plasma membrane. Therefore, we expected that disruption of a putative ER retention signal would result in the movement of ovine PGH synthase-1 from the ER

membrane to the plasma membrane. The converse has been reported by Tang *et al.* (131) with dipeptidyl peptidase IV (DPPIV), a type II integral plasma membrane protein. Addition of -KDEL to the C-terminus of DPPIV resulted in a change of localization from the plasma membrane to the ER membrane.

Mutations previously shown to disrupt functional ER retention signals (123,125,127,128,131) were introduced at the C-terminus of ovine PGH synthase-1. It has been demonstrated repeatedly that mutation of either glutamate or leucine of -KDEL retention signals results in loss of retention. Even a seemingly conservative change from leucine to valine is not tolerated in the consensus sequence. However, *cos*-1 cells transfected with PGH synthases -1 having various mutations in the -PTEL sequences showed no staining of the plasma membrane. Furthermore, media from *cos*-1 cells expressing native or mutant PGH synthases -1 showed no detectable PGH synthase-1 by Western blot analysis. Native and mutant PGH synthases -1 were only detected in the ER and NE. Because C-terminal mutations did not alter the subcellular locations of PGH synthase-1 and, based on assays of activity, the mutant proteins had near normal structures, we conclude that the -PTEL sequence is not required to target PGH synthase-1 to the ER.

The results obtained with the L600V mutant of PGH synthase-1 were surprising. Western blot analysis of two independent L600V mutants expressed in *cos*-1 cells showed that this mutation leads to an apparent loss of 9 kDa in molecular mass and a complete loss of enzyme activity. We were unable to account for the smaller mass of the L600V mutant based on either sensitivity to proteolytic digestion or lack of N-glycosylation. A decrease of 9 kDa in molecular mass corresponds to a loss of approximately 70 amino acids. Several reports have described PGH synthase splice variants (57,159), one of which encodes a protein lacking approximately 37 amino acids (159). We

speculate that the L600V mutation at the 3' end of the PGH synthase cDNA coding region promotes upstream splicing events which result in a variant protein of smaller molecular weight being expressed.

In summary, data from our experiments indicate that a mechanism other that the -KDEL retention pathway is responsible for retaining ovine PGH synthase-1 in the ER and NE. The protein structure of ovine PGH synthase-1 has been determined for residues 33-586. However, lack of crystalographic data for the C-terminal residues 587-600 suggests that the C-terminus is without definite secondary structure. X-ray crystal structure analysis has suggested that ovine PGH synthase-1 could associate with membranes via a group of planar amphipathic helices situated to expose hydrophobic residues to the luminal side of the ER membrane (36). Using this model to orient the molecule, we extrapolate that following residue 586, the unstructured C-terminal tail would project toward the ER membrane. Perhaps this toplogical arrangement renders the P/TEL C-terminal peptide inaccessible for neccessary interactions of the KDEL retention system. It will be important to determine if the proposed membrane binding domain composed of planar amphipathic helices, or some other domain of PGH synthase-1, is responsible for targeting the enzyme to the ER.

Acknowledgment

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CHAPTER 3

LOCALIZATION OF PGH SYNTHASE-2

Abstract

Polyclonal antisera specific for prostaglandin endoperoxide (PGH) synthases-1 and -2, respectively, were used to determine the subcellular locations of each PGH synthase isozyme in detergent-permeabilized mouse 3T3 fibroblasts by indirect immunocytofluorescence. Antiserum to PGH synthase-1 demonstrated a mottled pattern of cytoplasmic and perinuclear staining of both serum-starved and serum-stimulated 3T3 cells. This pattern of staining is consistent with the results of earlier studies which demonstrated that PGH synthase-1 is associated with the endoplasmic reticulum and NE of these cells. As expected, antibodies directed against a peptide unique to PGH synthase-2 failed to stain serum-starved cells, which lack appreciable levels of this second form of the enzyme. However, serum-stimulated 3T3 cells, which do express PGH synthase-2, showed the same pattern of staining with PGH synthase-2 antibodies as was observed with anti-PGH synthase-1 serum—mottled cytoplasmic staining and perinuclear staining. We conclude that the subcellular location of PGH synthase-2 is the same as PGH synthase-1 in murine 3T3 cells. Thus, the notable differences in the primary amino acid sequence—the signal peptide and the additional 18 amino acid C-terminal segment in PGH synthase-2—do not cause a change in localization. Colocalization of PGH synthases-1 and -2 implies that the source of arachidonate substrate, the site of PGH_2 and prostanoid formation, and the

mechanism of product transport from the inside to the outside of the cell are the same for these isozymes.

Introduction

PGH synthase is found in virtually all mammalian tissues (3) and catalyzes the conversion of arachidonic acid to prostaglandin endoperoxide H_2 (PGH_2) in a two step process carried out by cyclooxygenase and peroxidase activities. Prostaglandins and thromboxane A_2, the final products formed from the intermediate precursor PGH_2, are molecules involved in a variety of physiological roles such as inflammation (160,161), fluid volume homeostasis (162), ovulation (163-165), and mitogenesis (166,167). The current understanding of PGH synthase structure and function is based on data from studies with PGH synthase-1, originally isolated from sheep seminal vesicles (52,53). PGH synthase-1 is a 72 kDa hemoprotein that is membrane-associated and N-glycosylated (35,54,55,168,169).

The subcellular location of PGH-1 synthase was first determined by immunofluorescence in kidney tissue sections (90,170) to be in the endoplasmic reticulum and nuclear membrane. This result was later confirmed by immunoelectron microscopy of cultured mouse fibroblasts (91). Subsequent studies have continued to support the notion that, in general, PGH synthase-1 is localized to the endoplasmic and nuclear membranes. However, two additional locations of PGH synthase-1 have been reported. First, in 1983, Smith *et al.* demonstrated the presence of plasma membrane-associated PGH synthase-1 in arterial vascular smooth muscle—the single tissue positive for surface PGH synthase-1 of thirty-five different smooth muscle layers tested (171). Second, Weller *et al.* (172) recently described the association of PGH synthase-1 with non-membranous, structurally distinct lipid bodies. This is particularly interesting because lipid bodies represent a pool of arachidonate distinct from the prototypical sources of membrane phospholipids.

Recently, a gene encoding a second form of the enzyme, PGH synthase-2, has been cloned from chicken (57), mouse (58,59), and human (61) sources. Efforts to characterize this isozyme have been undertaken by several investigators. PGH synthase-2 differs from PGH synthase-1 in several ways. Comparison of the primary amino acid sequences shows that the signal peptides are dissimilar (64), and that there is a unique, C-terminal segment of 18 amino acids which is present only in PGH synthase-2. PGH synthase-2 also has a unique pattern of expression. In quiescent NIH 3T3 cells, constitutive levels of PGH synthase-2 mRNA (38,58,173) and PGH synthase-2 protein (68) are not detectable by Northern blot or Western blot analysis, respectively. However, stimulation by serum and growth factors rapidly induces transcription (38,58,59,61,64,173) and expression of PGH synthase-2 (68). The induction is transient and protein levels return to near basal levels within 8 hours of initial stimulation (68).

Experiments have shown that PGH synthase-2 is encoded by a distinct gene (61,174) and is differentially glycosylated to form a 70 and 72 kilodalton protein (84). PGH synthase-2 catalyzes cyclooxygenase and peroxidase reactions (174) and has a K_m for arachidonic acid similar to that of PGH synthase-1 (41). Both isozymes possess a N-terminal signal sequence peptide which is cleaved during protein maturation (54,64), and each has a putative endoplasmic reticulum retention signal at the C-terminus (128,133). This paper investigates the previously unanswered question of how the subcellular location of PGH synthase-2 compares to that of PGH synthase-1.

Materials and Methods

Preparation of Antibodies.

Isozyme-specific antisera for PGH synthase-2 was produced by immunizing rabbits with the synthetic 17-mer peptide, cys-tyr-ser-his-ser-arg-leu-asp-asp-iso-asn-pro-thr-val-leu-iso-lys, which was coupled to maleimide-activated keyhole limpet hemocyanin (KLH) (Pierce). This peptide corresponds to a unique region of PGH synthase-2 protein near the carboxy-terminus which is not present in PGH synthase-1; the amino-terminal cysteine was added to the synthetic peptide sequence to facilitate coupling to maleimide-activated KLH. Affinity-purified PGH synthase-2 antibodies were prepared according to Mumby *et al.* (175) after applying 10 mg of the synthetic peptide to a SulfoLink coupling gel (Pierce). Antisera to PGH synthase-1 was produced by immunizing rabbits with purified sheep vesicular gland PGH synthase (91).

Cell Culture.

NIH 3T3 mouse fibroblasts were cultured in Dulbecco's modified Eagle medium (DME) supplemented with 8% calf serum (Hyclone) and 2% fetal calf serum (Hyclone). After treatment with 0.25% trypsin, cells from a 60 to 80% confluent monolayer (100 X 20 mm plate) were diluted in 15 mls of serum-supplemented DME and pipetted onto sterile glass coverslips (22 X 22 mm) (Corning). Approximately 4 hours later, the coverslips were washed with PBS, and the adherent cells were grown in DME supplemented with 0.2% calf serum for 24 to 48 hours (serum-starved conditions). For growth under serum-stimulated conditions, starved cells were supplied with 16.7% fetal calf serum (1 ml fetal calf serum added to 5 ml of serum-starved medium) and incubated for 2 hours prior to immunofluorescence staining.

Immunofluorescence Staining.

Intracellular staining was performed at room temperature. Coverslips with cultured cells were washed in phosphate-buffered saline (PBS), fixed for 8 minutes in 2% formaldehyde in PBS, and washed in 10% calf serum in PBS. Coverslips were inverted onto 200 ml of a) PGH synthase-1 antiserum diluted 1:20 in PBS containing 0.2% saponin and 10% serum, or b) affinity-purified PGH synthase-2 antibodies (0.29 mg/ml) diluted 1:20 in PBS containing 0.2% saponin and 10% serum, and the samples were incubated for 1 hour. After washing in PBS containing 10% calf serum, the coverslips were incubated for 1 hour with a 1:40 dilution of fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (whole molecule) (Sigma) in PBS containing 0.2% saponin and 10% serum. Coverslips were then washed in PBS containing 10% calf serum, rinsed with PBS, and mounted on slides with Slowfade bleaching retardant (Molecular Probes). This procedure is essentially as described by Lippincott *et al.* (176). For a negative staining control, the affinity-purified PGH synthase-2 antibodies were incubated with the 17-mer antigen for 30 minutes prior to dilution and use on coverslips. The final concentration of peptide was 10 μM; the final dilution of purified antiserum was 1:20. Non-immune serum for PGH synthase-1 was obtained from a rabbit prior to immunization and diluted 1:20 in PBS before use.

Staining for cell-surface antigens was performed as above with the following exceptions: a) staining was performed at 4° C; b) saponin was *not* added to the antibody solutions; and c) fixation was performed *after* incubation with the secondary antibody. Coverslips were incubated with PGH synthase-1 antisera or PGH synthase-2 antibodies for 1 hour. The samples were rinsed in PBS containing 10% calf serum, and incubated with FITC-labeled goat anti-rabbit IgG. The cells were fixed for 8 minutes with 2%

formaldehyde in PBS and mounted on slides with Slowfade. For a positive control, the same staining procedure was performed using rat anti-transferrin receptor monoclonal (IgG) hybridoma (ATCC, TIB 219) supernatant (0.050 mg/ml) and FITC-conjugated goat anti-rat IgG (BMB). Approximately 8 mg of anti-transferrin receptor IgG per coverslip and a 1:40 dilution of anti-rat IgG in PBS were used as primary and secondary antibodies, respectively.

Confocal Microscopy.

A Meridian Instruments Insight bilateral scanning confocal microscope was used with an argon ion laser as the excitation source. A 100X objective lens and laser power of 30 to 50 mwatts were used for photography. Photographs were taken with Kodak Tri-X Pan 400 film with an exposure of 3 to 6 seconds.

Results and Discussion

Subcellular Localization of PGH Synthase-1.

PGH synthase-1 antiserum exhibited specificity for the form 1 isozyme under the denaturing conditions of Western blot analysis (68). However, the possibility of PGH synthase-1 antiserum cross-reacting with native confirmations of PGH synthase-2 could not be eliminated. Therefore, immunofluorescent staining for PGH synthase-1 was performed using serum-starved cells which do not express detectable levels of PGH synthase-2 as determined by Western blot analysis (68). (The pattern of PGH synthase-1 staining observed in serum-starved cells was identical to the PGH synthase-1 pattern observed in serum-stimulated cells (data not shown).) Serum-starved 3T3 cells were fixed with formaldehyde and then incubated sequentially in the presence of 0.2% saponin with antiserum to PGH synthase-1 and then FITC-labeled anti-rabbit IgG. As shown in Figure 13 A, cells stained using this procedure demonstrated immunofluorescent staining around the nucleus and mottled staining within the cytoplasm, a pattern of staining characteristic of antigens associated with the endoplasmic reticulum (177,178). As expected, when the staining procedure was performed using non-immune rabbit serum in place of the antiserum to PGH synthase-1, no immunofluorescent staining was observed (Figure 13 B). Staining of nonpermeabilized cells showed no plasma membrane staining for PGH synthase-1, while cell-surface staining was observed with a monoclonal antibody to the transferrin receptor, as expected (data not shown).

In an earlier study employing immunoelectron microscopy and this same anti-PGH synthase-1 serum, PGH synthase immunoreactivity was found to be associated with the nuclear membrane and the endoplasmic reticulum of murine 3T3 cells (91). Thus, the results of the present study employing

Figure 13. PGH synthase-1 immunofluorescence staining of mouse fibroblasts. NIH 3T3 cells were seeded onto coverslips (approximately 100 cells/coverslip) and starved for 24-48 hours in DME containing 0.2% calf serum. After 8 minutes of fixation with 2% formaldehyde in PBS, the coverslips were rinsed with PBS containing 10% calf serum and incubated 1 hour with a) PGH synthase-1 antiserum, or b) non-immune serum diluted in PBS containing 0.2% saponin and 10% calf serum. The coverslips were then incubated for 1 hour with FITC-conjugated goat anti-rabbit IgG diluted in the same solution as above. Coverslips were rinsed with PBS and mounted using a bleaching retardant compound. Immunofluorescence was photographed using the same exposure time in each case. Magnification, X 330.

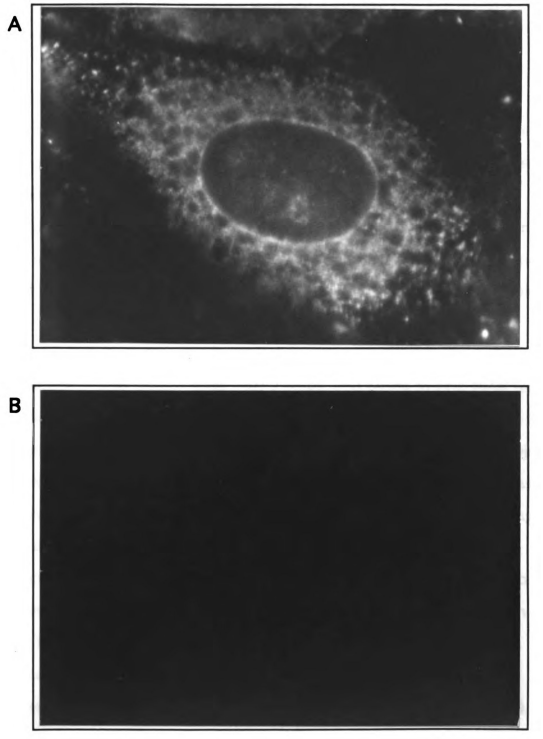

FIGURE 13

confocal microscopy are in accord with those obtained by immunoelectron microscopy.

Subcellular Localization of PGH Synthase-2.

A rabbit antiserum was prepared against a C-terminal peptide segment unique to murine PGH synthase-2, and the antibodies to this peptide were purified by affinity chromatography. In Western blot analysis, these antibodies were shown to be specific for PGH synthase-2 (68). Expression of PGH synthase-2 is a transient phenomenon initiated by serum stimulation (38,58,59,61,64,173). Serum-starved NIH 3T3 cells fail to express detectable levels of PGH synthase-2 as determined by Western blot analysis (68). However, following treatment with fetal calf serum, PGH synthase-2 levels are detectable after 1 hour, peak after 2 hours, and return to near basal levels after 8 hours (68). Therefore, to localize PGH synthase-2, serum-starved NIH 3T3 cells were stimulated with fetal calf serum for 2 hours and then subjected to immunofluorescent staining using the PGH synthase-2 antibodies. As shown in Figure 14 A, both perinuclear and mottled cytoplasmic staining were observed. Moreover, when the purified anti-peptide serum was preincubated with the peptide itself prior to staining stimulated cells, no staining was observed (Figure 14 B). As expected, no staining was observed using PGH synthase-2 antibodies with serum-starved cells (Figure 14 C). No plasma membrane immunofluorescence was observed using nonpermeabilized cells and PGH synthase-2 antibodies (data not shown).

These results indicate that PGH synthase-2 expressed in serum-stimulated NIH 3T3 cells is associated with the NE and the endoplasmic reticulum. Thus, PGH synthase-2 is found in association with the same membranes as PGH synthase-1 in murine fibroblasts. Further experiments to determine the

Figure 14. PGH synthase-2 immunofluorescence staining of mouse fibroblasts. NIH 3T3 cells were seeded onto coverslips (approximately 100 cells/coverslip) and starved for 24-48 hours in DME containing 0.2% calf serum. Cells were stimulated for 2 hours by the addition of fetal calf serum to a final concentration of 16.7% (A, B), or cells remained unstimulated (C). After fixation for 8 minutes with 2% formaldehyde in PBS, coverslips were rinsed with PBS and incubated for 1 hour with purified antibodies diluted in PBS containing 0.2% saponin and 10% calf serum: A, C) PGH synthase-2 antibodies, B) PGH synthase-2 antibodies preincubated with the peptide against which the antibody was generated (10 μM final concentration). The coverslips were then incubated for 1 hour with FITC-conjugated goat anti-rabbit IgG diluted in the same solution as above. Coverslips were rinsed in PBS and mounted using a bleaching retardant compound. Immunofluorescence was photographed using the same exposure time in each case. Magnification, X 330.

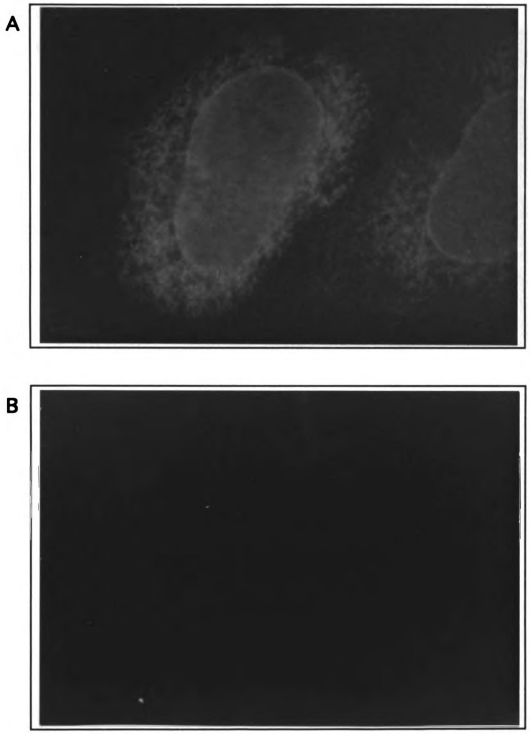

FIGURE 14

C

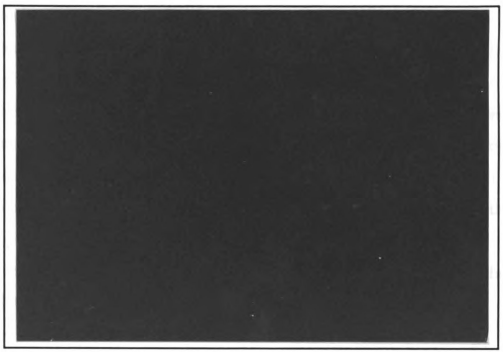

FIGURE 14 (cont'd)

extent of colocalization of the isozymes could investigate PGH synthase-2 localization in arterial smooth muscle, the only tissue reported to express PGH synthase-1 at the cell surface (171). Additionally, studies to determine if PGH synthase-2, like PGH synthase-1, is associated with lipid bodies are important to extend our understanding of the relationship of these enzymes.

In summary, the data presented here suggest that subcellular locations of PGH synthase-1 and -2 are the same in murine 3T3 cells. From this, we can conclude that the obvious differences in primary amino acid sequences of the signal peptides and the unique C-terminal 18 amino acid segment in PGH synthase-2 do not cause a change in location. Colocalization implies that the source of arachidonate substrate and the site of PGH_2 and prostanoid formation is the same for PGH synthase-1 and -2; and further, that the mechanism of prostanoid transport from the inside to the outside of the cell are the same for both isozymes.

Acknowledgment

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CHAPTER 4

INVESTIGATION OF THE PGHS-2 CASSETTE

Introduction

The work presented in chapter 2 showed that PGH synthase-1 and -2 are both localized to the ER and NE in murine NIH 3T3 fibroblasts. The isozymes are present on the same intracellular membranes, however, PGH synthase-2 immunofluorescence appears more intense on the NE (Figure 15) (93,179). Intense fluorescence staining of the perinuclear ring, more intense than the staining in the ER, is consistently observed in PGH synthase-2 stained cells, whereas the fluorescence intensity of the perinuclear ring and ER in PGH synthase-1 stained cells is similar. This observation suggests that distribution of the isozymes is different in these membrane systems.

Based on this observation the subcellular localization of murine PGH synthase-1 and -2 in 3T3 fibroblasts was re-examined using quantitative confocal immunofluorescence imaging microscopy (93). This work was designed to quantify the observed difference in NE localization of PGH synthase isozymes. The fluorescence intensity in the NE and ER was determined for each isozyme and compared. The fluorescence intensity of PGH synthase-2 staining of the NE was twice as concentrated as that of the ER. In constrast, PGH synthase-1 fluorescence intentsity was approximately the same in the NE and the ER. The same results were obtained in both human and bovine endothelial cells and in experiments using a second PGH

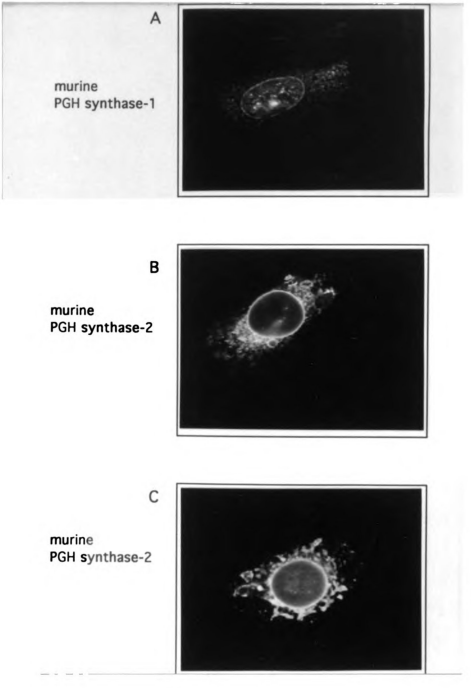


Figure 15. Subcellular localization of PGH synthase-1 and -2 in serum stimulated murine NIH 3T3 fibroblast. Murine NIH 3T3 fibroblasts were stimulated with serum for 2 hours and then processed for immunocytochemistry using purified isozyme-specific antisera as follows: A, anti-Leu274-Ala288 murine PGHS-1; B, anti-Ser582-Lys598 murine PGHS-2; C, anti-Gln569-Asn580 murine PGHS-2.

synthase-2 antiserum (93). Therefore, the localization of PGH synthase-2 is quantitatively different from PGH synthase-1. PGH synthase-2 is preferentially distributed, targeted, to the NE, while PGH synthase-1 is uniformly distributed in the ER and NE.

We reasoned that unique localization of PGH synthase-2 could be maintained by a unique protein structural domain of the second isozyme. As discussed in Chapter 1, the amino acid sequences of PGH synthase-1 and -2 are similar in most of the protein. However, one region of sequence divergence is found at the C-termini. Alignment of the murine isozyme sequences shows a 18 amino acid sequence at the PGH synthase C-terminus (PGHS-2 cassette) which is not found in PGH synthase-1 (Figure 16). The objective of this work was to determine whether the PGHS-2 cassette is involved in targeting the enzyme to the NE.

Figure 16. Comparison of the deduced amino acid sequences of murine PGH synthase-1 and murine PGH synthase-2. Murine PGH synthase-1 sequence in shown in the upper line, murine PGH synthase-2 in the lower line. The amino acid numbering of each isozyme is shown at the side. The signal peptides are in bold type. The sites of glycosylation are noted by an open triangle. The serine acetylated by aspirin is marked with circle. The residues important in catalysis are in bold type. The PGHS-2 cassette (Ala581-Lys598) near the C-terminus of PGH synthase-2 is in bold type. Epitopes used to generate isozyme specific antisera are underlined: Gln569-Asn580 and Ser582-Lys598 for PGH synthase-2; Leu274-Ala288 for PGH synthase-1.

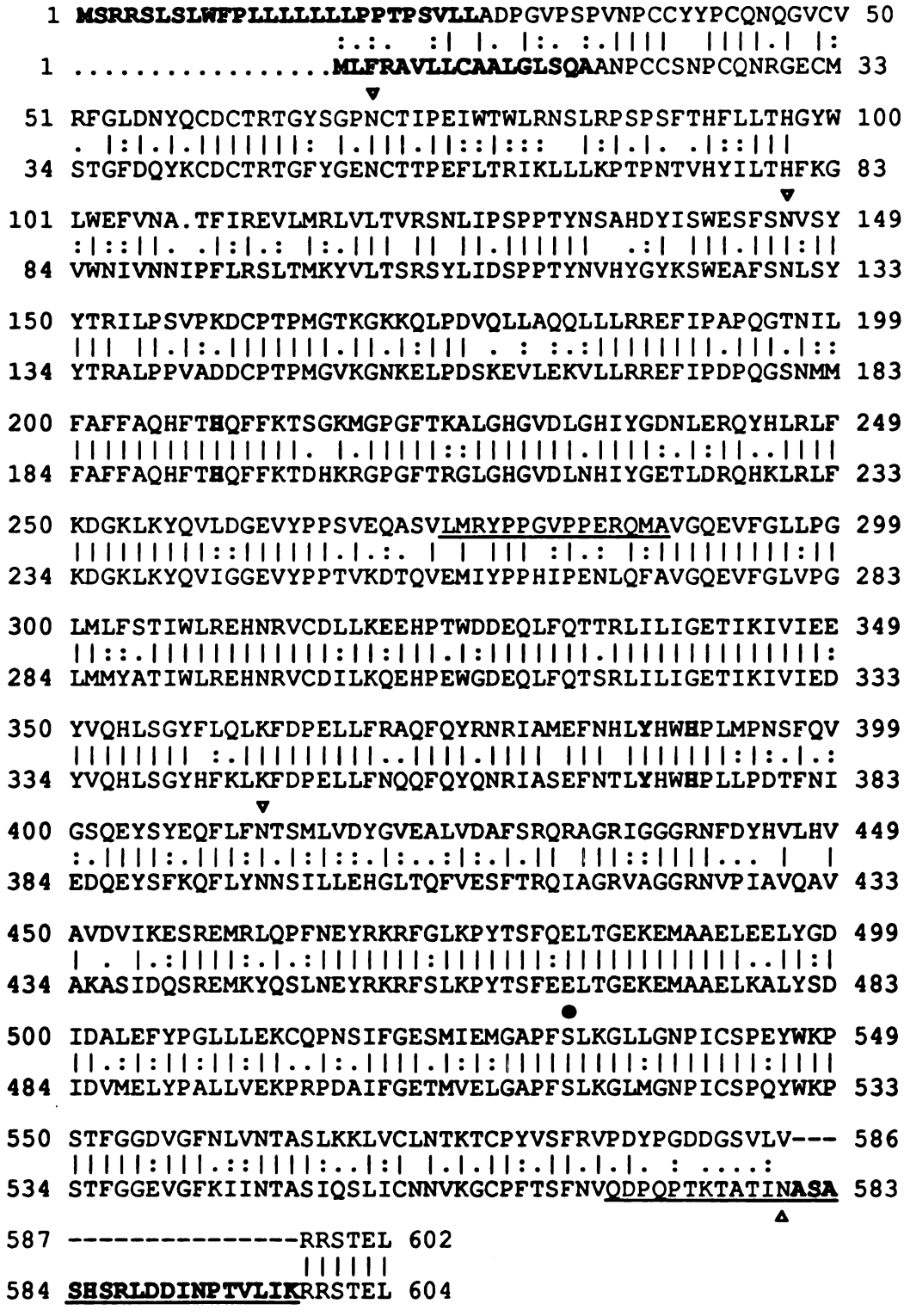


FIGURE 16

Materials and Methods

Materials

Many of the reagents used are described in Chapter 2 and 3. Materials used for microinjection are cited here: CELLocate coverslips were from Eppendorf North America. LabTech mulit-well culture dishes were from Nunc. Borosilicate glass capillary tubing was from World Precision Instruments.

Methods

Many of the methods used are described in Chapter 2 and 3. The conditions for cell culture of NIH 3T3 fibroblasts are described in chapter 3; for *cos*-1 cells, in chapter 2. The methods for mutagenesis, transient transfection, and Western blot analysis are described in Chapter 2. Cyclooxygenase activity was determined by polarographic oxygen electrode and is described in Chapter 2. The immunocytochemistry protocol for subcellular localization of PGH synthases was performed by 2% formaldehyde fixation and 0.2% permeabilization and is described in chapter 2 and 3. Details pertaining to the experiments in Chapter 4 are described below.

Mutagenesis

Murine PGH synthase-2 cDNA was subcloned into the *Not* I restriction site of M13mp48, a M13mp19 derivative with a modified polylinker. Deletion mutagenesis was performed using a 35 base synthetic oligonucleotide mutagensis primer that encodes the residues flanking the PGHS-2 cassette (Figure 17). The resulting mutant which lacks the PGHS-2 cassette was designated as PGH synthase-2Δcassette. Mutagenesis was performed using a Muta-gene kit patterned after the method of Kunkel (152). Native and mutant murine PGH synthase-2 cDNAs were subcloned into the *Not* I

restriction site of the pSVLN expression plasmid, a derivative of pSVL (153) with an altered polylinker, and sequenced to verify the mutations (151). Ovine PGH synthase-1 cDNA was subcloned into the *Sal* I restriction site of the pSVT7 expression plasmid (153).

Cell culture and transient transfection

Cos-1 cells were transfected using the DEAE dextran/chloroquine method. Murine PGH synthase-1 and -2 and PGH synthase-2Δcassette were transfected into *cos*-1 cells for preparation of microsomes or for immunocytochemistry. Transfections without plasmid (sham) were performed for negative controls. Cells for immunocytochemistry were cultured on CELLocate glass coverslips.

Isozyme-specific Antibodies:

A number of isozyme-selective antisera were used for immunocytochemistry. Those antisera previously described were generated from isozyme-specific peptides from the following protein regions: Ala25-Cys35 of ovine PGH synthase-1 (89); Leu274-Ala288 of murine PGH synthase-1 (93); and Ser582-Lys598 murine PGH synthase-2 (92). A second isozyme-specific antisera for murine PGH synthase-2 was produced by immunizing rabbits with the synthetic 13-mer peptide, Cys-Gln-Asp-Pro-Gln-Pro-Thr-Lys-Thr-Ala-Thr-Iso-Asn, which was coupled to maleimide-activated keyhole limpet hemocyanin (KLH) (Pierce). This peptide which corresponds to the amino acids (Gln569-Asn580) immediately upstream of the PGHS-2 cassette is not similar in sequence to the analogous residues in murine PGH synthase-1 (Figure 16). The amino-terminal cysteine was added to the synthetic peptide sequence to facilitate coupling to maleimide-activated KLH. Affinity-purified PGH synthase-2 antibodies were prepared according to Mumby *et al.* (175) after

applying 10 mg of the synthetic peptide to a SulfoLink coupling gel (Pierce). Purified antiserum is immunoreactive with both native murine PGH synthase-2 and PGH synthase-2-Δcassette, but is not immunoreactive with murine PGH synthase-1 as determined by Western blot analysis (Figure 18).

Western Blot Analysis

Microsomes were prepared from murine PGH synthase-1, murine PGH synthase-2, and PGH synthase-2Δcassette transfected *cos*-1 cells as described in chapter 3. Microsomes prepared from cells transfected with no DNA (sham) were used as controls. Microsomal protein preparations were subjected to Western blot analysis (10 μg protein/lane). Visualization of native and mutant protein was performed using murine PGH synthase-2-specific antiserum and ECL kit reagents.

K_m determination

K_m values were determined by measuring cyclooxygenase activity using arachidonate ranging from 2 to 100 μM . The K_m value was calculated from Lineweaver-Burk analysis.

Prostaglandin Synthesis

Prostaglandin product formation from [^{14}C] arachidonate by transfected *cos*-1 cells was performed as previously described (37). *Cos*-1 cells transfected with murine PGH synthase-1, murine PGH synthase-2, PGH synthase-2-Δcassette, or without plasmid (sham) were harvested in ice-cold PBS by scraping the culture dish with a spatula. Cells were resuspended in DMEM and incubated with [^{14}C] arachidonate at 37° C for 40 min. The cells were collected by centrifugation, and arachidonate products were isolated from the

supernatant by chloroform extraction following acidification. The lipid products were separated by thin-layer chromatography in benzene:dioxane:acetic acid: formic acid (82:14:1:1; v:v:v), and visualized by exposure to X-ray film for 40 hours. Radioactive bands were identfied by comparison with prostaglandin standards.

Microinjection

Cos-1 cells and 3T3 fibroblasts were cultured on CELLocate glass coverslips in 100 x 20 mm tissue culture dishes, and maintained in DMEM supplemented with 8% fetal calf serum, 2% bovine calf serum for *cos*-1 cells and 3T3 fibroblasts, or 0.2% bovine calf serum for quiescent 3T3 fibroblasts. CELLocate coverslips are glass coverslips etched with a 175 μm alphanumeric grid to enable precise relocation of individual (microinjected) cells. After cell attachment, coverslips were transferred to multi-well glass slides, LabTech chambers, and covered with 1.5 ml of appropriate media. Cells were at subconfluent densities at the time of injection. Nuclei were injected with cesium chloride-purified plasmid DNA at 1 mg/ml in PBS containing 1 mM $MgCl_2$. Microinjection was performed using an Insight bilateral scanning confocal microscope (Meridian Instruments, Okemos, MI). The micropipette was mounted on the Insight confocal microscope with an IMT2-SFY inject attachment, and maneuvered with a micromanipulator (Narishige). This system is designed for vertical injection of samples, a micropipette angle that minimizes interference of the phase contrast optics used to view the injection. Injection of plasmid solutions was controled by hydraulic pressure using a glass syringe and tubing connected to the micropipette. Cells were viewed with 20X objective for injections. Native and mutant PGH synthases

were expressed by direct microinjection of plasmid DNA into nuclei using the methods of Capecchi (180) as described by Wozniak (147).

Micropipettes were pulled from thin-wall borosilicate glass capillaries with internal filament (1 mm outer diameter, 0.75 inner diameter) using a Sutter Instrument P-87 Brown-flaming micropipette puller. Successful microinjection was obtained with the following instrument parameter values: Heat, 715 ; Pull 70; Velocity, 90; Time, 250; and Pressure, 400.

Fluorescence Confocal Microscopy

An Insight Bilateral scanning confocal microscope (Meridian Instruments, Okemos, MI) was used with an argon ion laser as the excitation source (181). Laser power settings between 50 and 75% were used depending on intensity of staining.

Results

Expression and catalytic activity of PGH synthase-2-Δcassette

Site-directed deletion mutagenesis was performed to generate a murine PGH synthase-2 mutant lacking the PGHS-2 cassette (PGH synthase-2-Δcassette) (Figure 17). Deletion of the PGHS-2 cassette removes 18 amino acids from the protein and eliminates a site of glycosylation at Asn580. Native murine PGH synthase-2 and mutant PGH synthase-2Δcassette cDNAs were subcloned into the pSVL expression vector. To determine the effect of the mutations on the structural and catalytic properties of the enzyme, we performed Western blot analysis (Figure 18) and prostaglandin product analysis (Figure 19). Western blot analyses of *cos*-1 microsomal protein preparations showed that PGH synthase-2Δcassette had an altered electrophoretic mobility as compared with native PGH synthase-2 (74 and 72 kDa). PGH synthase-2Δcassette migrated at an apparent molecular mass of 68 kDa, which was consistent with the estimated loss of mass resulting from deletion of the PGHS-2 cassette (18 amino acids and one N-linked carbohydrate moiety). Deletion of the PGHS-2 cassette disrupts the glycosylation consensus sequence (Asn-Ala-Ser) of Asn580. Glycosylation of native PGH synthase-2 at this site is about 50% efficient, which results in two protein species that migrate as 74 and 72 kDa by SDS PAGE (84). PGH synthase-2Δcassette migrates as a single 68 kDa species as expected in the absence of differential glycosylation at Asn580.

Cos-1 cells transfected with native murine PGH synthase-1 and -2 and mutant PGH synthase-2-Δcassette were incubated with [^{14}C] arachidonate, and prostaglandin products were separated by thin-layer chromatography. *Cos*-1 cells expressing PGH synthase-2-Δcassette generated prostaglandin products

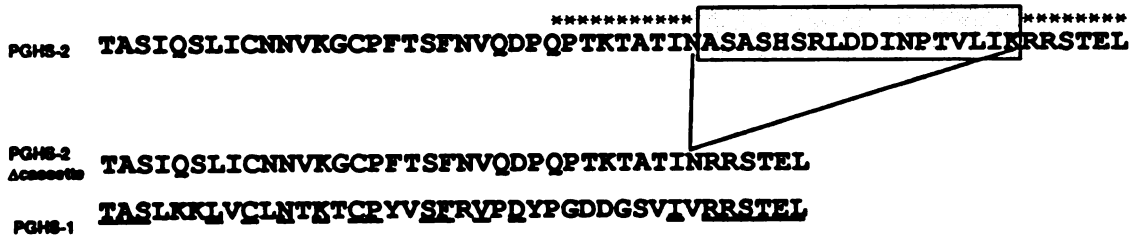


Figure 17. Mutagenesis scheme for PGH synthase-2Δcassette. The C-terminal regions of murine PGH synthase-2 (PGHS-2), PGH synthase-2Δcassette (PGHS-2Δcassette), and murine PGH synthase-1 (PGHS-1) are aligned. The PGHS-2 cassette which was deleted in mutagenesis is marked in a stipled box. Residues corresponding to the oligonucleotide used in mutagenesis are marked with overlined asterisks. Identical residues in murine PGH synthase-1 and PGH synthase-2Δcassette are underlined.

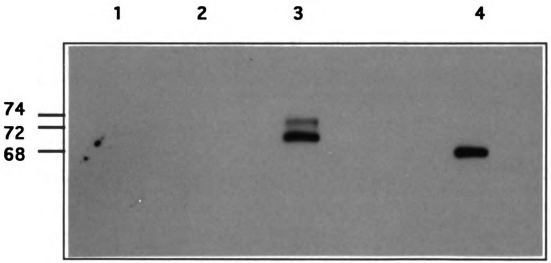


Figure 18. Isozyme-specific immunoreactivity of purified anti-Gln569-Asn580 murine PGH synthase-2 antisera, and expression of PGH synthase-2Δcassette. Isozyme-specificity of antisera generated against residues Gln-569-Asn580 of murine PGH synthase-2 was tested by Western blot analysis. *Cos*-1 cells were transiently transfected with pSVLN expression plasmids encoding PGH synthase cDNAs. Microsomal protein was subjected to Western blot analysis using purified antisera generated against residues Gln-569-Asn580 of murine PGH synthase-2. Microsomal samples (10 μg protein/lane) from transiently transfected *cos*-1 cells are as follows: transfected without plasmid, Lane 1; murine PGH synthase-1, lane 2; murine PGH synthase-2, lane 3; and PGH synthase-2Δcassette, lane 4.

(PGE2, PGF2α, PGD2) comparable to those observed with native enzymes (Figure 19), indicating that PGH synthase-2-Δcassette retains cyclooxygenase and peroxidase activity.

To further investigate the effect of PGHS-2 cassette deletion, the K_m for native murine PGH synthase and PGH synthase-2-Δcassette were compared. Microsomes prepared from transfected *cos*-1 cells were used in cyclooxygenase activity measurements to determine the K_m values for the native and mutant enzymes. The K_m values with arachidonate were similar, 3.3 μM for PGH synthase-2-Δcassette and 2.5 μM for native murine PGH synthase-2. These values are also similar to that previously reported for native murine PGH synthase-2, 2.5 μM arachidonate (41) . We conclude that deletion of PGHS-2 cassette is permissible with relatively minor changes in the conformation of murine PGH synthase-2.

Subcellular localization of PGH synthase-2Δcassette in transiently transfected cos-1 cells

Immunocytochemistry was performed to determine whether deletion of the PGHS-2 cassette altered the preferential localization of PGH synthase-2 to the NE. Intracellular staining of *cos*-1 cells transfected with native murine PGH synthase-1 and -2 cDNAs and mutant PGH synthase-2-Δcassette cDNA was performed. In all cases staining was observed in the ER and NE (Figure 20). Quantitation of fluorescence intensity in the ER and NE was difficult because of the unusual morphology of the transfected *cos*-1 cells. Often the *cos*-1 nucleus is asymmetrically positioned in the cytoplasm with a portion of the nuclear perimeter touching the plasma membrane. In previous studies using 3T3 fibroblasts, the nucleus is usually centered in the cytoplasm and fluorescence can be quantitated more easily. Quantitation was also difficult in

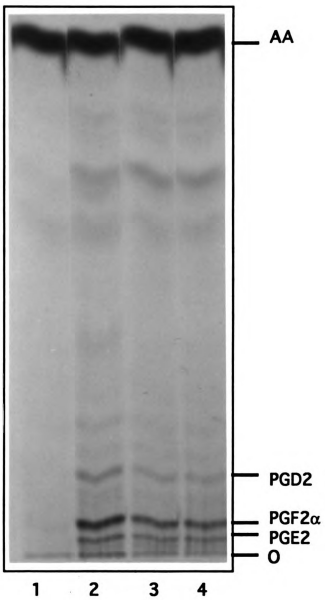


Figure 19. Prostaglandins synthesis in *cos*-1 cells transiently transfected with native and mutant PGH synthases. *Cos*-1 cells were transiently transfected with pSVLN expression plasmids encoding PGH synthase cDNAs. Transfected *cos*-1 cells were harvested and incubated with [^{14}C] arachidonate Prostaglandins products were separated by thin-layer chromatography. Lane 1, no plasmid, Lane-2 murine PGH synthase-1; Lane 3, murine PGH synthase-2; Lane 4, PGH synthase-2Δcassette. Prostaglandin species, arachidonic acid (AA) and the origin (O) are identified on the right edge.

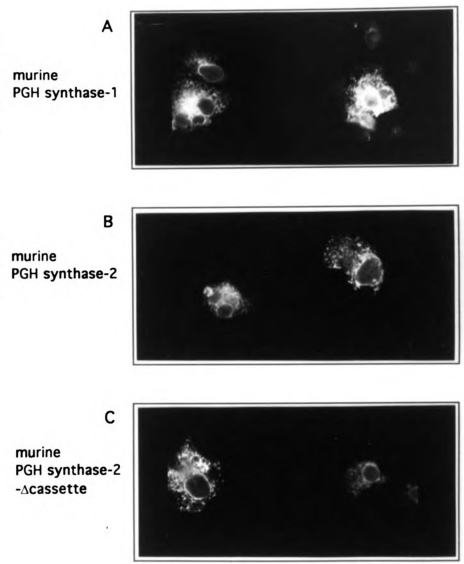


Figure 20. Expression of native and mutant PGH synthases in transiently transfected *cos*-1 cells. *Cos*-1 cells were transiently transfected with pSVLN expression plasmids encoding PGH synthase cDNAs and stained with anti-PGH synthase antisera: A, murine PGH synthase-1 stained with anti-Leu274-Ala288 PGHS-1; B, murine-PGH synthase-2 and C, PGH synthase-2Δcassette stained with anti-Gln569-Asn580 PGHS-2.

cos-1 cells due to areas of intense cytoplasmic immunofluorescence (Figure 20).

The ratio of NE to ER fluorescence intensities (NE/ER ratio) determined for native murine PGH synthase-1 and -2 and PGH synthase-2-Δcassette in transfected *cos*-1 cells were 1.4, 1.2, and 1.0 respectively. The PGH synthase-1 and -2 NE/ER ratios do not agree with those observed in 3T3 cells (PGH synthase-1, approximately 1.0, PGH synthase-2, approximately 2.0) (93) suggesting that localization of PGH synthase-2 overexpressed in cells may be aberrant. We conclude that transient transfection of *cos*-1 cells is not a suitable system for localization experiments regarding PGH synthase-2 NE targeting. To overcome this limitation, I next attempted to express these enzymes in 3T3 cells by microinjection of their cDNAs.

Subcellular localization of PGH synthase-2Δcassette by 3T3 microinjection in cos-1 and 3T3 cells

Localization experiments were performed by direct injection of plasmid cDNAs in cell nuclei. Preliminary microinjection experiments to establish the parameters of the protocol were conducted using *cos*-1 cells and native ovine PGH synthase-1 cDNA in the pSVT7 expression plasmid. Ovine PGH synthase-1 cDNA plasmid was microinjected in *cos*-1 nuclei. The cells were then maintained in culture for various times, and then processed for immunofluorescence staining. Microinjected cells from 5, 10, 18, 24 and 48 hours post-injection were analyzed. Ovine PGH synthase-1 protein expressed from microinjected plasmid was localized to the ER and NE (Figure 21). The number of cells expressing microinjected plasmid was greatest for samples incubated 5 hours post-injection, and no expression was observed for cells cultured more than 24 hours. We speculate that the viability of microinjected

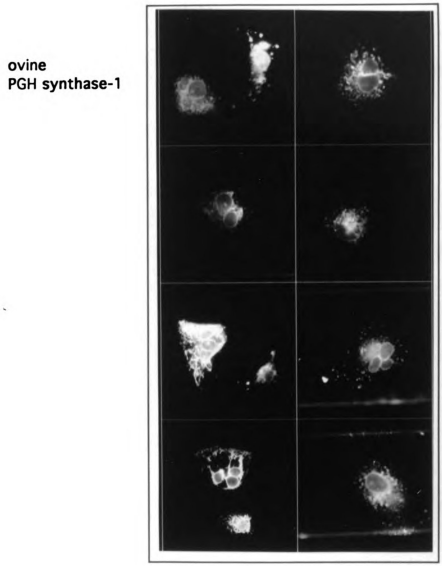


Figure 21. Subcellular localization of ovine PGH synthase-1 in microinjected *cos*-1 cells. *Cos*-1 cells were microinjected with ovine PGH synthase-1 and stained with anti-Ala25-Cys35 ovine PGHS-1. Cells from several experiments are shown.

cells is compromised, and after longer incubation periods microinjected cells die and detach from the coverslip. We conclude that incubation periods as short as 5 hours post-injection are sufficient for detectable expression of cDNAs in the pSVT7 expression plasmid. Unfortunately, microinjected *cos*-1 cells exhibited the same morphology transfected *cos*-1 cells, a morphology not conducive to quantitation. In addition, very few injected cells expressed the injected plasmid (see further discussion below). Therefore, I next attempted to microinject 3T3 cells, the cell line used for the quantified subcellular localization of the native murine PGH synthase isozymes.

Microinjection of native ovine PGH synthase-1 in pSVT7 and native murine PGH synthase-2 in the pSVL expression plasmid was performed in growing and quiescent 3T3 fibroblasts nuclei. Injected cells were cultured for 5 hours and then processed for immunocytochemistry. Staining of ovine PGH synthase-1 and murine PGH synthase-2 was observed in the ER and NE (Figure 22). These experiments demonstrate that expression of microinjected pSVT7 and pSVL expression plasmids in growing and quiescent cells is detectable as early as 5 hours post-injection. Importantly, we demonstrate that these plasmids are expressed in quiescent 3T3 cells which is critical to our experimental design (see discussion below).

Although expression of microinjected plasmids was achieved, the efficiency of expression was substandard. An average of one percent or fewer of injected cells were found to express PGH synthase. The most successful experiment yielded nine positively expressing cells out of approximately 500 cells injected. Many expressing cells appeared damaged or multinucleated (Figure 21, Figure 22). We conclude that our microinjection technique will need to be improved considerably to make this technique a feasible approach for localization studies.

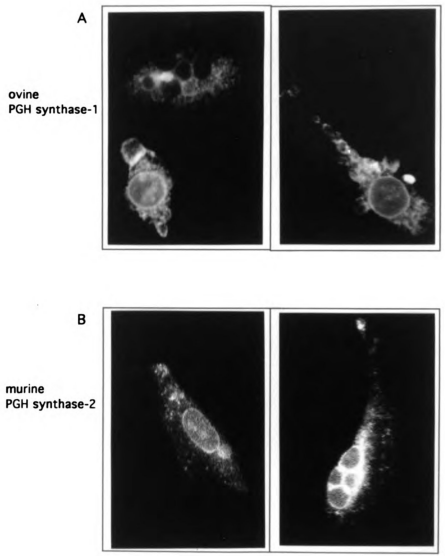


Figure 22. Subcellular localization of ovine PGH synthase-1 and murine PGH synthase-2 in microinjected quiescent murine NIH 3T3 fibroblasts. Quiescent NIH 3T3 cells were microinjected with (A) ovine PGH synthase-1, stained with anti-Ala25-Cys35 ovine PGHS-1 and (B) murine PGH synthase-2, stained with anti-Gln569-Asn580 murine PGHS-2.

Discussion

Although both PGH synthase isozymes are present in the ER and NE, recent work has shown that PGH synthase-2 is preferentially distributed in the NE (93). Intense staining of the perinuclear ring which is greater than the intensity of the ER has been shown for PGH synthase-2 in 3T3 fibroblasts, bovine arterial endothelial cells, and human umbilical vein endothelial cells (93). PGH synthase-1 staining in these cells is of similar intensity in the ER and NE. Staining intensities were quantitated and expressed as a ratio of NE to ER fluorescence intensities (NE/ER ratio) for PGH synthase-1 and-2. The PGH synthase-2 NE/ER ratio is approximately 2, whereas the NE/ER ratio for PGH synthase-1 is about 1. Because immunoreactivity, or fluorescence intensity, is roughly proportional to the mass of protein present, the NE/ER ratio results suggest that PGH synthase-2 is concentrated in the NE. This is unlike PGH synthase-1 which is uniformly distributed in the two membrane systems.

It seems likely that the unique localization pattern of PGH synthase-2 could be related to a unique structural domain in the protein. We were interested in identifying the putative protein determinant responsible for concentrating PGH synthase-2 in the NE. Murine PGH synthase-1 and -2 share significant similarity in protein sequence (Figure 16). However, at the C-terminus of PGH synthase-2 there is a unique sequence of 18 residues (Ala581-Lys598). The peptide sequence abruptly interrupts the well conserved isozyme sequence alignment, and its sequence is not similar to any other reported protein sequence (GenBank sequences). This 18 amino acid sequence (PGHS-2 cassette) which is found only in PGH synthase-2 was identified as a candidate for a PGH synthase-2 NE targeting domain. Site directed mutagenesis was performed to delete the PGHS-2 cassette from murine PGH

synthase-2, and I have attempted to determine what effect this deletion has on PGH synthase-2 localization.

No obvious structural or catalytic changes occured following deletion of the PGHS-2 cassette from PGH synthase-2. Cyclooxygenase activity, formation of prostaglandins, and affinity for arachidonate are similar for PGH synthase-2-Δcassette and native murine PGH synthase-2. Thus the PGHS-2 cassette is not involved in enzyme catalysis. Information inferred from the ovine PGH synthase-1 crystal structure supports this interpretation (36). The protein structure for the C-terminal tail of ovine PGH synthase-1, Arg586 to Leu600 (ovine PGH synthase-1 numbering), is absent from the crystal structure, which suggests that this region of ovine PGH synthase-1 is disordered. Together these results suggest that the PGHS-2 cassette, like the PGH synthase-1 C-terminal tail, does not interact significantly with other structural domains of the PGH synthase protein.

Using *cos*-1 cells expressing native and mutant enzymes we were unable to localize murine PGH synthase-2 or PGH synthase-2-Δcassette. The NE/ER ratio calculated for murine PGH synthase-1 and -2 and PGH synthase-2-Δcassette in transfected cells did not agree with NE/ER ratios of the native enzymes endogenously expressed in murine 3T3 fibroblasts. Two technical limitations precluded our use of the *cos*-1 system. First, high levels of overexpressed protein in *cos*-1 cells caused very intense staining in certain regions of the cytoplasm. Our method of NE/ER ratio quantitation cannot accommodate such non uniform staining of cytoplasmic areas immediately surrounding the nucleus. Second, *cos*-1 nuclei were frequently positioned near the plasma membrane of the cell, rather than in the center of the cell; the NE was bordered by cytoplasm for less than the entire perimeter. Because accurate calculation of the NE/ER ratio is not feasible in *cos*-1 cells, we

conclude that transient transfection in this system is not suitable for our localization studies. It is also possible that *cos*-1 cells, which lack endogenous PGH synthase, lack the proper targeting machinery for differential distribution of PGH synthase-2, or that the targeting machinery is disrupted by overexpression or by the process of transfection.

A strategy employing microinjection of PGH synthase cDNA expression plasmids in 3T3 fibroblasts was developed as an alternative approach for localization experiments. This system has several advantages: expression plasmids are introduced without transfection treatments, and 3T3 fibroblasts have demonstrated differential localization of PGH synthase-2 previously (93). We modeled our experiments after the work of Wozniak and Blobel in the determination of the targeting domain of gp210, a glycoprotein of the nuclear pore complex (147). In their experiments mutant cDNAs carried in the pSVL expression plasmid were microinjected in 3T3 fibroblast, and localization was determined by immunocytochemistry.

3T3 fibroblasts constitutively express murine PGH synthase-1 and transiently express murine PGH synthase-2. Therefore, it was necessary to manipulate the experimental conditions so that protein expressed from microinjected plasmids could be distinguished from endogenous protein expression. The problem of endogenous murine PGH synthase-2 expression was addressed by culturing cells in low serum medium (0.2% serum); 3T3 fibroblasts grown under these conditions do not express PGH synthase-2. To circumvent the problem of endogenous murine PGH synthase-1 expression, ovine PGH synthase-1 was used in microinjection experiments; species-selective antisera were used to detect only the transfected ovine PGH synthase-1.

A series of control experiments were performed to establish the technique of microinjection and the proper microinjection conditions for our system. This work included developing micropipettes appropriate for microinjection, determining the optimal time of expression following microinjection, and determining whether pSVT7 and pSVL expression plasmids are expressed in quiescent cells. Expression of microinjected plasmids was achieved for native ovine PGH synthase-1 and native murine PGH synthase-2 in *cos*-1 cells (Figure 21) and quiescent 3T3 fibroblasts (Figure 22). Importantly, this work demonstrates that our system of microinjection is a valid approach for studying the localization of PGH synthase in quiescent 3T3 fibroblasts.

A significant limitation in these experiments was the very low percentage of injected cells found to express PGH synthase. In all experiments, one percent or fewer of the cells injected expressed protein. This compares with 20-50% expressing cells reported by others using a similar system (R. Wozniak, personal communication). In addition microinjections were successful only occasionally; in my final series of experiments, one out of four days injecting produced cells that expressed protein.

Improvement in the efficiency of microinjection will be necessary to make this experimental approach practical for regular use. My experience suggests that the micropipettes and the method of solution injection are very important components of the system. It is critical that the micropipette is small enough to avoid excessive damage to the NE, and that the injection of plasmid solution be very carefully controlled to avoid injection of too large a volume (R. Wozniak, personal communication). Cell viability following injection is the bottleneck in this technique. Refining these two parameters is likely to dramatically improve microinjection efficiency.

In summary, the work presented here provides initial characterization of PGH synthase-Δcassette. The mutant enzyme retains cyclooxygenase and peroxidase activities, and produces prostaglandins similar to native PGH synthase, and removal of the PGHS-2 cassette does not alter the enzyme affinity for substrate. These data demonstrate that the PGHS-2 cassette is not involved in catalysis. Subcellular localization studies demonstrate that pSVT7 and pSVL expression plasmids are expressed in quiescent 3T3 cells, 5 hours post-injection. Improvement of the microinjection system will be necessary to obtain sufficient sample sizes for NE/ER ratio calculation. This system will be useful for assaying the PGHS-2 cassette and other domains of interest in localization of PGH synthase-2.

CHAPTER 5

SUBCELLULAR LOCALIZATION OF CYTOSOLIC PHOSPHOLIPASE A_2

Abstract

Cytosolic phospholipase A_2 ($cPLA_2$) is induced by a wide variety of stimuli to release arachidonic acid, the precursor of the potent inflammatory mediators prostaglandins and leukotrienes. Specifically, $cPLA_2$ releases arachidonic acid in response to agents that increase intracellular Ca^{2+}. *In vitro* data has suggested that these agents induce a translocation of $cPLA_2$ from the cytosol to the cell membrane, where its substrate is localized. Here, we use immunofluorescence to visualize the translocation of $cPLA_2$ to distinct cellular membranes. In CHO cells that stably overexpress $cPLA_2$, this enzyme translocates to the nuclear envelope upon stimulation with the calcium ionophore A23187. The pattern of staining observed in the cytoplasm suggests that $cPLA_2$ may also translocate to the endoplasmic reticulum. We find no evidence for $cPLA_2$ localization to the plasma membrane. This translocation is dependent on the calcium-dependent phospholipid binding (CaLB) domain, as a CaLB deletion mutant of $cPLA_2$ (ΔCII) fails to translocate in response to Ca^{2+}. In contrast, $cPLA_2$ mutated at Ser505, the site of mitogen-activated protein (MAP) kinase phosphorylation, translocates normally. This observation, combined with the observed phosphorylation of ΔCII, establishes that these two mechanisms function independently to regulate $cPLA_2$. The effect of these mutations on $cPLA_2$ translocation was confirmed by subcellular fractionation. Each of these

mutations abolishes the ability of cPLA2 to release arachidonic acid, establishing that cPLA2-mediated arachidonic acid release is strongly dependent on both phosphorylation and translocation. These data help to clarify the mechanisms by which cPLA2 is regulated in intact cells and establish the nuclear envelope as a primary site for arachidonic acid production in the cell.

Introduction

The 85 kD cytosolic phospholipase A_2 ($cPLA_2$), which selectively releases arachidonic acid from the *sn*-2 position of membrane phospholipids, is crucial to the initiation of the inflammatory response. $cPLA_2$ activity is stimulated by a wide variety of agents, including the proinflammatory cytokines interleukin 1 (182,183) and tumor necrosis factor (184), macrophage colony-stimulating factor (184), thrombin (185,186), ATP (185), mitogens (29,187-189) and endothelin (190). The release of arachidonic acid is the rate-limiting step in the generation of prostaglandins and leukotrienes, the proinflammatory eicosanoids. Cleavage of arachidonyl-containing phospholipids also results in the release of lysophospholipid, the precursor of the inflammatory mediator platelet-activating factor (191).

$cPLA_2$ is expressed in many cell types. Many of these are associated with the inflammatory response, such as monocytes (184), neutrophils (192), and synovial fibroblasts (193). It is also expressed in a wide variety of tissues, including kidney, spleen, heart, lung, liver, testis and hippocampus (194). This diverse pattern of expression is consistent with accumulating evidence that in addition to its role in inflammation, $cPLA_2$ also participates in the signaling of such diverse processes as platelet activation (21,186), tumor necrosis factor-induced cytotoxicity (195), and proliferation (187,189,196).

$cPLA_2$ activity is regulated both transcriptionally and post-translationally. Post-translational activation is thought to occur by two mechanisms. One mechanism involves agonist-induced MAP kinase phosphorylation of $cPLA_2$, resulting in stimulation of its instrinsic enzymatic activity (22,197). The second involves the Ca^{2+}-dependent translocation of $cPLA_2$ from the soluble to the membrane fraction of cells (20,198,199), allowing $cPLA_2$ access to its arachidonyl phospholipid substrate. As discussed below, the results

presented in this study establish that both mechanisms are critical for the stimulation of cPLA2-induced arachidonic acid release.

In vitro studies have strongly suggested that the membrane-binding function of cPLA2 resides in its Ca^{2+}-dependent phospholipid-binding (CaLB) domain (20,200), a region similar to the CII domain of protein kinase C. This domain has been shown to be necessary and sufficient for Ca^{2+}-dependent membrane binding (20,200). In this study we characterize the translocation of cPLA2 in intact cells and establish that this enzyme translocates to the nuclear envelope. Our data also suggests that cPLA2 translocates to the endoplasmic reticulum. Deleting the CaLB domain abolishes the ability of cPLA2 to translocate to these membranes, whereas a mutation at the MAP kinase phosphorylation site has no effect on translocation. Either of these mutations prevents cPLA2-induced arachidonic acid release. These data, together with recent reports localizing several arachidonic acid-metabolizing enzymes to the nuclear envelope and endoplasmic reticulum (92,201-203), establish these membranes as important sites of arachidonic acid production and metabolism in the cell.

Materials and Methods

Cell Culture and Antibodies

CHO cells were maintained as previously described (185). Monoclonal antibody 1.1.1 (22) was raised against human cPLA2 purified from E5-CHO cells and was used for staining of parental and cPLA2-overexpressing CHO cells. Polyclonal antibody 7905, generated against cPLA2 produced in *Escherichia coli*, was used for immunoblotting (20). PGHS-1-specific polyclonal antibody was generated against the N-terminus of ovine PGHS-1, residues Ala-25 to Cys-35 (89). E-selectin monoclonal antibody was generated against soluble human E-selectin (204).

Immunofluorescence Staining

Cells grown on coverslips were rinsed with serum-free media prior to a 2 min treatment with 2 μM calcium ionophore A23187 (Sigma). Treated and untreated cells were washed twice briefly with TBS containing 0.01% Triton X-100. During all washes and incubations throughout this procedure, all A23187-treated cells were incubated in the presence of 1 mM $CaCl_2$, to lessen the chance that translocated cPLA2 would detach from membranes. Cells were fixed for 2 min at room temperature in a 50:50 mixture of acetone and methanol. After 2 brief washes, blocking was performed in TBS/20% goat serum for 1 hr at 37°C. Cells were incubated with primary antibody (150 mg/ml cPLA2 antibody, 100 mg/ml E-selectin antibody, or a 1:20 dilution of PGHS-1 antibody) for 1 hr at 37° and washed once briefly followed by 2 x 10 min washes. Second antibody incubation was performed for 1 hr at 37°C using a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse or -rabbit antibody and washed as above. After a brief wash with

water, coverslips were inverted onto slides with Slowfade bleaching retardant (Molecular Probes).

Fluorescence Confocal Microscopy

Subcellular localization of cPLA2 was visualized by fluorescence confocal microscopy. An Insight Bilateral scanning confocal microscope (Meridian Instruments, Okemos, MI) was used with an argon ion laser as the excitation source (181). The laser was used at a power ranging from 25-75 mW, depending on the level of fluorescence intensity. All images were photographed with a 100X objective. Untreated and A23187-treated samples for a given cell line were analyzed using identical instrument settings.

Fractionation and Immunoblotting

Cells were starved overnight in serum-free media and treated with 2 μM A23187. After washing with TBS, cells were collected by scraping and lysed in 1 ml lysis buffer (20 mM Hepes, pH 7.4/ 1 mM EGTA/ 0.34 M sucrose/ 10 μg/ml leupeptin/ 2 mM PMSF/ 5 mM DTT) by Parr bombing, 600 psi for 5 min. Lysates were centrifuged for 1 hr at 100,000 x *g* and 5 mg of protein from supernatant and pellet fractions was electrophoresed by SDS PAGE. Proteins were transferred to nitrocellulose, blotted with anti-cPLA2 polyclonal antibody 7905 (20), and developed by chemiluminescence after incubation with HRP-conjugated protein A (Amersham).

Arachidonic Acid Release

Cells were plated onto 12-well cluster dishes (Costar) at 1.5 x 10^5 cells per well in growth medium. After a 20 hr incubation, the medium was removed and replaced with 0.5 ml of a medium (GIBCO) containing 0.5 μCi of

[^{3}H]arachidonic acid (100 Ci/mmol; New England Nuclear) and incubated for another 20 hr at 37°C. The cells were then washed three times with medium containing 0.1% bovine serum albumin and incubated with A23187 for 10 min. The medium was removed, and radioactivity was determined by scintillation counting.

Results

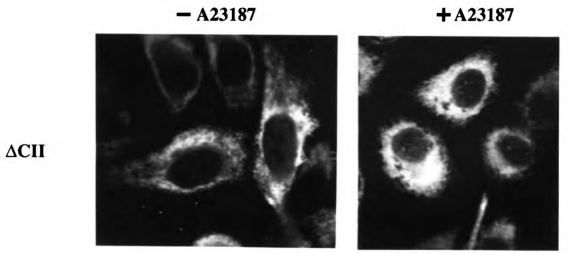
Ca^{2+} Ionophore Induces cPLA2 Translocation to the Nuclear Envelope

cPLA2 can be induced to associate with natural membranes in the presence of physiologically relevant Ca^{2+} levels *in vitro* (20,198). This Ca^{2+}-dependent translocation is thought to reflect a translocation that occurs *in vivo* when cells are stimulated with agents that increase intracellular Ca^{2+}. As the translocation of cPLA2 in response to Ca^{2+} has not yet been characterized in intact cells, the membrane site(s) of arachidonic acid release by cPLA2 has not been identified. To address this issue, indirect immunofluorescence was performed using E5-4, a CHO cell line that overexpresses human cPLA2 (185) (Figure 23 A). In resting cells, cPLA2 was found distributed throughout the cytoplasm. In contrast, after treatment with the Ca^{2+} ionophore A23187, significant cPLA2 staining appeared in a discrete ring surrounding the nucleus. This suggests that an increase in cytosolic Ca^{2+} induces a translocation of cPLA2 from the cytosol to the nuclear envelope.

Interestingly, staining of A23187-treated E5-4 cells was consistently brighter than that seen in untreated cells (quantitated in Figure 23 B). This is most likely due to a greater loss of soluble cPLA2 than membrane-bound cPLA2 throughout the staining procedure. A similar interpretation was offered for the results observed upon immunogold labeling of the arachidonic acid-metabolizing enzyme 5-lipoxygenase (5-LO), which also translocates to the nuclear envelope in response to A23187 (202). In that study, 5-LO was undetectable in unstimulated cells but was apparent at the nuclear envelope after ionophore treatment. Alternatively, binding of cPLA2 to membranes may result in a better exposure of the monoclonal antibody epitope, resulting in more efficient antibody binding. In either case, the observed increase in cytoplasmic staining intensity suggests that cPLA2 may translocate not only to

Figure 23. Differential localization of cPLA$_2$ in untreated and Ca^{2+} ionophore-stimulated cells. CHO cells overexpressing wildtype cPLA$_2$ were stimulated with 2 μM Ca^{2+} ionophore (+A23187) for 2 min or left untreated (-A23187). (A) Immunofluorescence was performed using a monoclonal antibody specific for cPLA$_2$ and a fluorescein-conjugated second antibody. Confocal microscopy reveals a translocation of cPLA$_2$ to the nuclear envelope (magnification, X330). As discussed in the text, punctate staining was observed in the cytoplasm in some experiments, consistent with translocation to the endoplasmic reticulum. (B) Quantitative fluorescence imaging of cytoplasm and nuclear envelope. The intensity of immunofluorescent staining was quantified by measuring fluorescence before and after ionophore treatment in equivalent areas of the cytoplasm and nuclear envelope.

A


− A23187
+ A23187
ΔCII

B

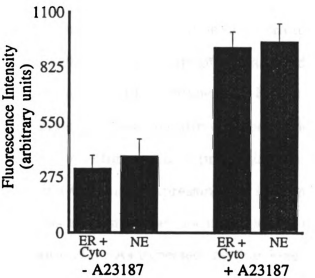

Fluorescence Intensity (arbitrary units)
1100
825
550
275
0
ER + Cyto
NE
ER + Cyto
NE
- A23187
+ A23187

FIGURE 23

the nuclear envelope but also to a cytoplasmic membrane structure, most likely the endoplasmic reticulum. Indeed, in some experiments ionophore treatment resulted in an increase in punctate cytoplasmic fluorescence characteristic of endoplasmic reticulum staining.

The plasma membrane was consistently found to be devoid of cPLA2 staining. To ensure that fixation had not disrupted the integrity of the plasma membrane, CHO cells overexpressing the plasma membrane protein E-selectin were stained. As shown in Figure 24, E-selectin antibody was clearly able to label the plasma membrane. No labeling of the nuclear envelope was observed in these cells. As a control for the specificity of the anti-cPLA2 monoclonal antibody, immunofluorescence was also performed on the parental CHO cells used for these studies. Only faint staining was observed (Figure 24), similar to that seen when the monoclonal antibody was eliminated from the staining protocol (data not shown). The lack of endogenous cPLA2 staining is likely to be due both to the low level of cPLA2 expressed in these cells and the failure of this antibody to recognize murine (and by inference, hamster) cPLA2 efficiently. Figure 24 also shows staining of CHO cells overexpressing prostaglandin-endoperoxide synthase-1 (PGHS-1), which metabolizes arachidonic acid to prostaglandin. Similar staining results were obtained with a CHO line expressing the isoform PGHS-2 (not shown). Both PGHS isoforms and have been localized to the endoplasmic reticulum and nuclear envelope (92). As expected, neither E-selectin nor PGHS-1 staining patterns were affected by ionophore treatment.

CaLB, but not Ser505 Phosphorylation, is Required for Translocation

In cell-free systems, the CaLB domain is required for the association of cPLA2 with membranes (200). To confirm the importance of the CaLB

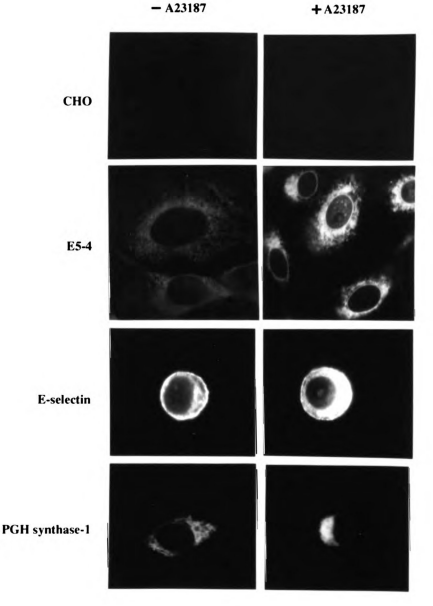


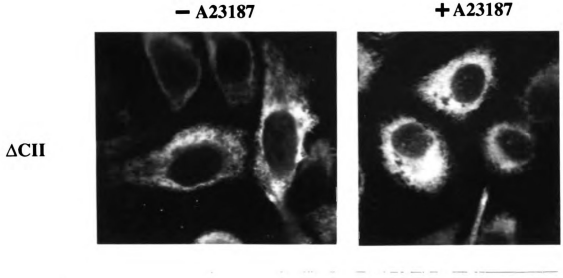
Figure 24. Confocal microscopy of CHO cells overexpressing various proteins. Indirect immunofluorescence was performed on untransfected CHO cells and stable CHO cell lines expressing cPLA2, E-selectin, and PGHS-1, with (+) and without (-) A23187 treatment.

domain for cPLA2 translocation in intact cells, immunofluorescence was performed on a CHO line (ΔCII) that overexpresses cPLA2 lacking the CaLB domain (amino acids 1-134) (200). As shown in Figure 25 A, no increase in nuclear envelope staining was observed, indicating that this truncated cPLA2 is unable to translocate in response to ionophore. Consistent with this result, ionophore did not induce an increase in staining intensity in these cells (Figure 25 B). The effect of a mutation at the MAP kinase phosphorylation site, Ser505, was also investigated. MAP kinase phosphorylation at this residue has previously been shown to increase the intrinsic enzymatic activity of cPLA2 (22,197). To determine whether this phosphorylation was involved in regulating translocation, immunofluorescence staining of CHO cells expressing SA505-cPLA2, containing a serine-to-alanine mutation at this residue, was performed. The translocation of SA505-cPLA2 in response to ionophore was indistinguishable from that observed with wildtype cPLA2 (Figure 26). The increase in staining intensity seen with wildtype cPLA2 upon inophore treatment was also observed in the SA505 line, further supporting the ability of this mutant to translocate to the membrane.

To confirm that deletion of the CaLB domain, but not the mutation of Ser-505, abolished cPLA2 translocation, fractionation was performed with cells expressing these mutants. After ionophore treatment, cell lysates were centrifuged at 100,000 x *g* and the soluble and particulate fractions immunoblotted for cPLA2. As shown in Figure 27, both cPLA2 and SA505 redistributed to the particulate fraction upon ionophore treatment, whereas ΔCII did not. The insert shown below the ΔCII portion of the figure represents a longer exposure of the blot, revealing the translocation of endogenous cPLA2 in the ΔCII line. These data establish that phosphorylation at Ser505 is not required for cPLA2 translocation.

Figure 25. Translocation of cPLA$_2$ is dependent on the CaLB domain. (A) ΔCII cells, which stably express cPLA$_2$ lacking the CaLB domain, show similar staining in untreated (-) and ionophore stimulated (+) cells. Staining and visualization are the same as described in the legend to Figure 23. (B) Quantification of fluorescence intensity. Staining intensity was measured as described in Figure 23 B.

A


− A23187
+ A23187
ΔCII

B

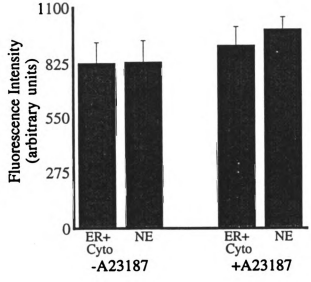

1100
825
550
275
0
Fluorescence Intensity (arbitrary units)
ER+ Cyto
NE
ER+ Cyto
NE
-A23187
+A23187

FIGURE 25

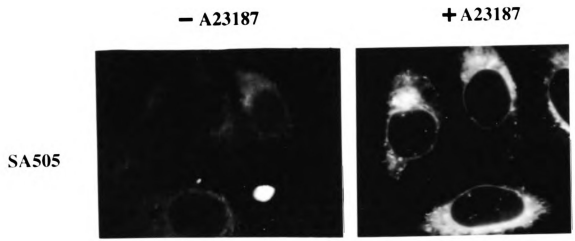


Figure 26. Translocation of SA505-cPLA$_2$. Cells stably overexpressing SA505-cPLA$_2$, which contains a serine-to-alanine mutation at the Ser505 MAP kinase phosphorylation site, were probed with antibody to cPLA$_2$. Indirect immunofluorescence was performed as described in the legend to Figure 23. As seen with wildtype cPLA$_2$ (Figure 23), ionophore (+A23187) induces the translocation of SA505-cPLA$_2$ to the nuclear membrane as well as an increase in overall fluorescent staining intensity.

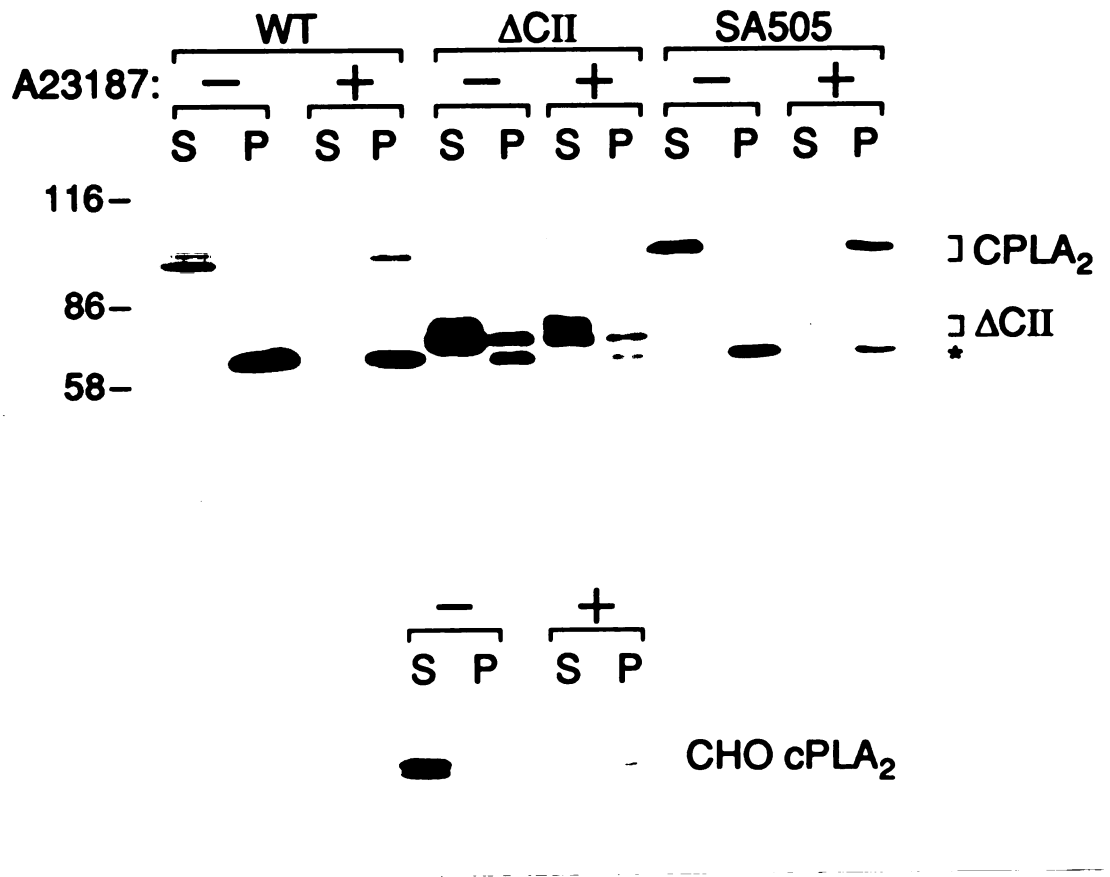


Figure 27. Fractionation of lysates from wildtype, ΔCII and SA505 cells. Cells overexpressing each cPLA2 construct were stimulated with 2 μM ionophore for 10 min (+) or left untreated (-). Lysates were spun at 100,000 x *g*, and 5 μg of protein from the supernatant (S) and pellet (P) fractions electrophoresed on SDS PAGE. Proteins were transferred to nitrocellulose and immunoblotted for cPLA2. Development was by chemiluminescence. The positions of full length cPLA2 and ΔCII are indicated. The insert below the ΔCII portion of the blot represents a longer exposure of these lanes. Endogenous cPLA2 can be seen to translocate normally in the ΔCII line. * indicates a cross-reactive species of unknown origin.

Conversely, translocation is not required for Ser505 phosphorylation, as evidenced by the presence of a cPLA2 doublet in the ΔCII but not the SA505 line. The upper band of the cPLA2 doublet has previously been established to be a result of phosphorylation at Ser505 (22). These data establish that translocation and phosphorylation occur independently of each another.

Both Translocation and Ser505 Phosphorylation are Required for Stimulation of Arachidonic Acid Release

In order to investigate the relative importance of translocation and Ser505 phosphorylation for cPLA2-mediated arachidonic acid release, the amount of arachidonic acid liberated upon A23187 treatment was measured in E5-4, ΔCII, SA505 and parental CHO cells (Figure 28). The level of arachidonic acid released from stimulated E5-4 cells was consistently at least 2 to 3 times greater than that released from parental CHO cells. As shown in Figure 28 A, cells expressing ΔCII did not show enhanced arachidonic acid release in response to A23187 relative to CHO cells. Similarly, SA505-expressing cells showed no increase in arachidonic acid release over the parental line (Figure 28 A). The observation that each of these mutations, separately, can prevent arachidonic acid release establishes that both translocation and Ser505 phosphorylation are indispensable to cPLA2 function in intact cells.

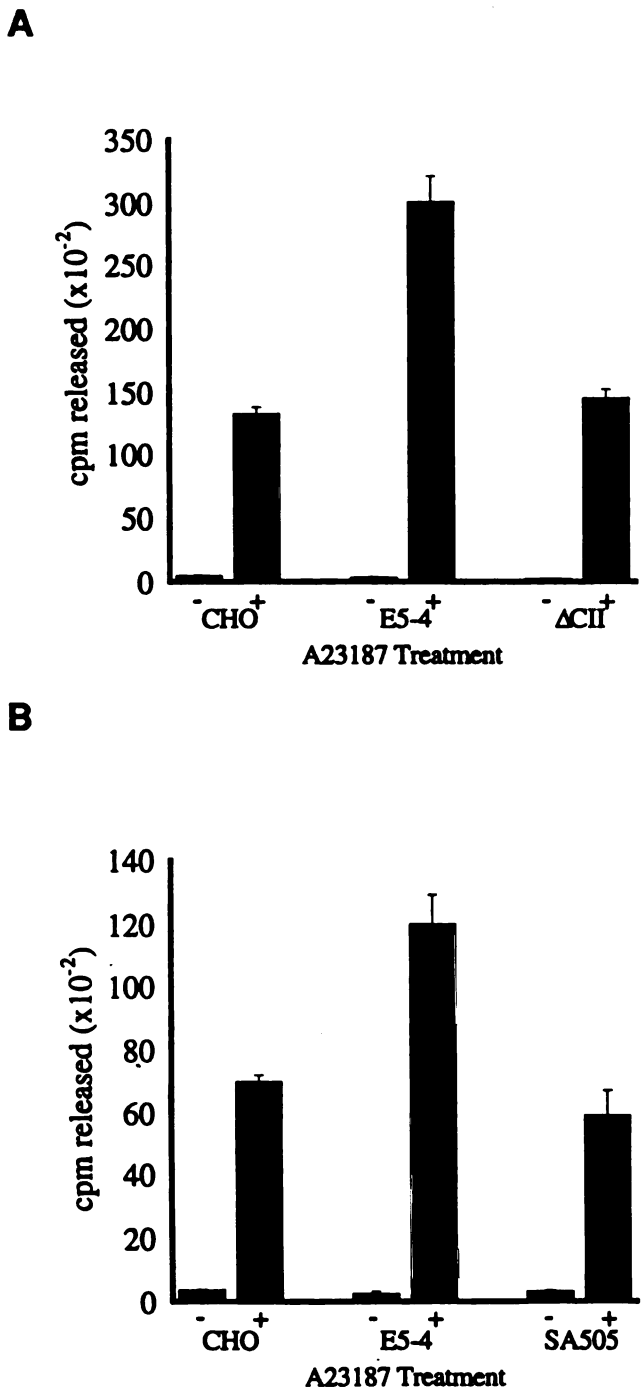


Figure 28. Effect of ΔCII and SA505 mutations on arachidonic acid release. Cells were labeled with [^{3}H]arachidonic acid for 20 hr. After washing, cells were stimulated with 2 μM A23187 (+) or left untreated (-) for 10 min, and radioactivity was measured in the media by scintillation counting. The levels of arachidonic acid released from ΔCII (A) and SA505 (B) are compared with parental CHO cells and E5-4.

Discussion

Cellular Localization of cPLA2

The data presented here establish that in intact cells, cPLA2 is induced to translocate to membranes in response to a rise in intracellular Ca^{2+}. Specifically, cPLA2 binds to the nuclear envelope. The increase in cytoplasmic staining intensity seen upon ionophore treatment suggests that cPLA2 also binds to a cytoplasmic membrane structure. The identity of this cytoplasmic membrane is not yet known, although we suggest that cPLA2 binds both the nuclear envelope and the endoplasmic reticulum, consistent with the localization of PGHS-1 and-2 (92). 5-lipoxygenase, which metabolizes arachidonic acid to leukotrienes, as well as its activating protein, FLAP, are localized to the nuclear envelope (202). These results, taken together, implicate the nuclear envelope and endoplasmic reticulum as the primary sites for arachidonic acid production and metabolism in the cell. Consistent with this, we note a complete absence of cPLA2 staining at the plasma membrane.

Translocation of cPLA2 is abolished upon deletion of the CaLB domain but not upon mutation of Ser505. This suggests that translocation and phosphorylation regulate cPLA2 independently, a conclusion supported by the observation that ΔCII is phosphorylated in CHO cells (as evidenced by the doublet in Figure 27). Both translocation and phosphorylation are critical for A23187-induced arachidonic acid release. The observation that these regulatory mechanisms function independently strongly supports a model in which phosphorylation at Ser505 serves primarily to activate the enzymatic activity of cPLA2 (22,197), and translocation allows access of the enzyme to its substrate.

This discussion focuses on the localization of cPLA2 to the nuclear envelope and endoplasmic reticulum, although the identity of the cytoplasmic membrane(s) to which cPLA2 binds has not yet been unequivocally established. How cPLA2 is selectively localized to these sites is not known. The translocation of cPLA2 to the nuclear envelope is consistent with previous data indicating that arachidonic acid is preferentially released from the nuclear envelope, as observed in pulse-chase experiments using [^{14}C]arachidonate-labeled HSDM$_1$ C$_1$ cells stimulated with bradykinin (205). Interestingly, electron microscopic studies using [^{3}H]arachidonic acid have shown that the nuclear membrane is the preferred site of initial arachidonic acid incorporation in these cells (206). Arachidonic acid is also incorporated rapidly into the endoplasmic reticulum; transit to the plasma membrane is slow. This study shows that although arachidonic acid is preferentially incorporated at particular sites, it becomes evenly distributed throughout the cell. Thus a correlation exists between the cellular localization of cPLA2 and the sites at which arachidonic acid is *initially* incorporated into the cell. However, the significance of this correlation is not known.

Several arachidonic acid-independent mechanisms could also explain the localization of cPLA2. A "docking protein" might bind cPLA2 at the nuclear membrane and endoplasmic reticulum, although it is known that such a protein is not essential, since Ca^{2+} induces cPLA2 membrane binding in the absence of protein (200). It is unlikely that a spatially-localized release of Ca^{2+} is responsible for the targeting of cPLA2, since our experiments revealed no translocation of cPLA2 to the plasma membrane with A23187, whose effects are thought to include the triggering of a Ca^{2+} influx across the plasma membrane. Another possibility is that some feature of the plasma membrane *excludes* cPLA2, and cPLA2 simply translocates to all cellular membranes

which it is capable of binding. Interestingly, the plasma membrane is known to be enriched in sphingolipids compared to the nuclear envelope and endoplasmic reticulum. It is conceivable that the increase in phospholipid packing density that results from a high sphingolipid content may prevent cPLA2 binding. Consistent with this idea, Leslie and Channon (198) have shown that both the activity and calcium sensitivity of cPLA2 are inhibited by the increased substrate packing density induced by sphingolipids. Clearly, a better understanding of the lipid and protein components of distinct cellular membranes will greatly facilitate our understanding of the mechanism(s) governing the localization of cPLA2.

Coupling of cPLA2 Induced Arachidonic Acid Release to PGHS

As discussed above, the localization of cPLA2 is similar to that reported for PGHS-1 and -2, which metabolize arachidonic acid to prostaglandins. PGHS-1 is expressed constitutively and is thought to perform certain physiological "housekeeping" functions. PGHS-2, whose expression is induced by cytokines, has been implicated in inflammation (48,49). It is tempting to speculate that cPLA2-mediated release of arachidonic acid couples primarily to PGHS-2, since both enzymes are inducible by a wide variety of agonists. Indeed, antisense oligonucleotide inhibition of cPLA2 has been shown to decrease the level of endotoxin-stimulated PGE_2 in monocytes (without affecting PGHS-2 activity) (207). This is significant because PGE_2 release in endotoxin-stimulated monocytes has been shown to be dependent on PGHS-2 activity even in the presence of PGHS-1 (32), suggesting a coupling between cPLA2 and PGHS-2. Studies in bone marrow-derived mast cells demonstrate the coupling of PGHS-1 and -2 to different stimuli (31), further supporting the idea that the PGHS isoforms participate in different signaling pathways and

may metabolize arachidonic acid from distinct pools. Experiments using an antisense oligonucleotide to the group II PLA_2 suggest that in macrophages stimulated with endotoxin and platelet-activating factor, different PLA_2 enzymes participate in different phases of arachidonic acid release (208). These data, taken together, suggest that different stimuli may induce distinct PLA_2 enzymes, which may themselves each couple to a specific PGHS isoform.

The presence of $cPLA_2$ in the nuclear envelope and (most likely) the endoplasmic reticulum correlates with the localization of both PGHS-1 and PGHS-2. However, a recent study that more closely examines the localization of these isoforms reveals that their cellular distributions overlap but are not identical. PGHS-1 was shown to be equally distributed in the endoplasmic reticulum and nuclear envelope, whereas PGHS-2 was twice as concentrated in the nuclear envelope as in the endoplasmic reticulum (93). Localization of the cyclooxygenase/peroxidase activity of these isoforms using a histofluorescent staining method revealed an even more striking difference. The fluorescent product formed from PHGS-1 activity was found in the cytoplasm, whereas that formed from PGHS-2 was detected in both the cytoplasm and within the nucleus. The colocalization of $cPLA_2$ protein and PGHS-2 activity at the nuclear membrane is consistent with a coupling between these enzymes. However, as $cPLA_2$ also appears to translocate to the endoplasmic reticulum, where both PGHS isoforms are located, a role in releasing arachidonic acid to PGHS-1 is also possible. Although the mechanisms of arachidonic acid transfer between the enzymes in this cascade have not yet been elucidated in detail, the colocalization of $cPLA_2$ with PGHS and 5-LO is certain to ensure the efficient utilization of arachidonic acid upon

agonist stimulation, consistent with a central role for cPLA2 in the agonist-induced biosynthesis of prostaglandins and leukotrienes.

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CONCLUSION

Distinct biological roles for PGH synthase isozymes

There is an increasing number of observations which suggest, when they are considered together, that PGH synthase isozymes have distinct biological roles. One observation is the unique patterns of isozyme expression. PGH synthase-1 is present constitutively which affords the cell a pathway for immediate synthesis of prostaglandins in response to hormonal signals associated with maintenance of physiological homeostasis. PGH synthase-2 prostaglandin synthesis is possible only after induction of the enzyme by an appropriate stimulus. Many of the extracellular signals which induce PGH synthase-2 are stimuli of mitogenesis or inflammation. A second observation is that prostaglandin biosynthetic capacity increases only 1.5-2 fold after induction of PGH synthase-2 (30,69,74) . Furthermore, the kinetic properties of PGH synthase-1 and -2 are very similar. It seems unlikely that PGH synthase-2, which has a similiar affinity for substrate, is induced soley to supplement PGH synthase-1 synthesis of PGH2.

Functional separation of isozymes is likely to occur at many levels. Evidence suggests that arachidonate is stored in isozyme-specific pools (31,32). In fibroblasts and macrophages, phorbol ester and LPS treatment induce PGH synthase-2 expression and mobilize archidonate. However, constitutive PGH synthase-1 does not utilize this supply of arachidonate for substrate (32). This suggests that PGH synthase-1 utilizes a unique pool of arachidonate which is separate from the pool mobilized by PGH synthase-2 inducers. In bone

marrow-derived mast cells prostaglandin synthesis from endogenous arachidonate occurs by two paths: a cytokine/PGH synthase-2 pathway and a IgE/PGH synthase-1 pathway (31). It is appears that these pathways are segregated by specific coupling of a specific agonist with a specific arachidonate pool.

Arachidonate pools may result from different phospholipase activities such as cytosolic PLA2 and secretory PLA2, or different sources such as LDL (27,28), lipid bodies (172), and membranes. The co-localization of cPLA2 and PGH synthase-1 and -2 to the ER and NE demonstrates cPLA2 is in a suitable cellular location to provide arachidonate to PGH synthase isozymes. Whether cPLA2 arachidonate release is channeled specifically to one of the isozymes remains to be seen.

Differential subcellular localization is another potential mechanism for functional separation of PGH synthase isozymes. While PGH synthase isozymes are localized to the same membrane systems, concentration of PGH synthase-2 at the NE is unique (93). It has also been shown that PGH synthase enzyme activity is uniquely distributed. PGH synthase-2 products are concentrated in the nucleus, while PGH synthase-1 products are mostly in the cytoplasm (93). Taken together this suggests that the biosynthetic pathways are separated by subcellular distribution of enzyme and products.

Whether it be different substrate pools, stimulus coupling, and/or localization of PGH synthase protein, it is expected that isozyme segregation is in some way related to protein structure. There are several regions of protein structural distinction between the isozymes. PGH synthase-2 has a unique 18 amino acid sequence near the C-terminus. We report here a mutant murine PGH synthase-2 that lacks this region, PGH synthase-2Δcassette, which is catalytically similar to the native enzyme. In addition we have generated an

antiserum that is immunoreactive with PGH synthase-2Δcassette in intact cells. These tools will be useful to further investigate the role of this unique PGH synthase-2 domain. Other regions in the isozyme amino acid sequences are distinct. The signal peptides of the isozyme are of different sequence and length. The membrane binding domains of the isozymes account for the regions of least sequence similarity (aside from the unique N- and C-termini) (209) . Isozyme specific protein structures such as these could impart unique functions to the isozyme itself or allow unique associations with other proteins in the prostaglandin biosynthetic pathway. Future studies focusing on these regions of the protein will provide insight into the biological distinction of PGH synthase-1 and -2.

LIST OF REFERENCES

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1. Smith, W. L., and Laneuville, O. (1994) in *Prostaglandin Inhibitors in Tumor Immunology and Immunotherapy*. (Harris, J. E., Braun, D., and Anderson, K. M., eds), pp. 1-40, CRC Press, Baca Raton.

2. McGiff, J. C. (1991) Cytochrome P-450 metabolism of arachidonic acid. *Annu. Rev. Pharmacol. Toxicol.*, **31**, 339-369.

3. Smith, W. L. (1987) in *CRC Handbook of Eicosanoids: Prostaglandins and Related Lipids* (Willis, A. L., ed) Vol. 1A, pp. 175-184, CRC Press, Boca Raton.

4. DeWitt, D. L. (1991) Prostaglandin endoperoxide synthase: regulation of enzyme expression. *Biochem. Biophys. Acta*, **1083**, 121-134.

5. Smith, W. L. (1986) Prostaglandin biosynthesis and its compartmentation in vascular smooth muscle and endothelial cells. *Annu. Rev. Physiol.*, **48**, 251-262.

6. Smith, W. L. (1992) Prostanoid biosynthesis and mechanisms of action. *Am. J. Physiol.*, **263**, F181-F191.

7. Smith, W. L. (1989) The eicosanoids and their biochemical mechanisms of action. *Biochem. J.*, **259**, 315-324.

8. Moncada, S. R., Gryglewski, R., and Vane, J. R. (1976) An enzyme isolated from artieries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature*, **263**, 663-665.

9. Arita, H., Nakano, T., and Hanasaki, K. (1989) Thromboxane A_2: Its generation and role in platelet activation. *Prog. Lipid Res.*, **28**, 273-301.

10. Hamberg, M., Svensson, J., and Samuelsson, B. (1975) Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Natl. Acad. Sci. USA*, **72**, 2994-2998.

11. Kuehl, F. A., and Egan, R. W. (1980) Prostaglandins, arachidonic acid, and inflammation. *Science*, **210**, 978-984.

12. Sewing, K.-F., and Beinborn, M. (1990) in *Advances in Prostaglandin , Thromboxane, and Leukotriene Research* (Samuelsson, B., Dahlen, S.-E., and Hedqvist, P., eds) Vol. 20, pp. 138-145, Raven Press, New York.

13. Coleman, R. A., Smith, W. L., and Narumiya, S. (1994) International union of pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharm. Rev.*, **46**, 205-229.

14. Cook, H. W. (1991) in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance, D. E., and Vance, J., eds) Vol. 20, pp. 141-169, Elsevier, New York.

15. Rittenhouse-Simmons, S., and Deykin, K. (1981) in *Platelets in Biology and Pathology* (Gordon, J. C., ed) Vol. 2, pp. 349-371, Elsevier/North Holland Biomedical Press, Amsterdam.

16. Irvine, R. F. (1982) How is the level of free arachidonic acid controlled in mammalian cells? *Biochem. J.*, **204**, 3-16.

17. Burgoyne, R. D., and Morgan, A. (1990) The control of free arachidonic acid levels. *TIBS*, **15**, 365-366.

18. Kudo, I., Marakami, M., Hara, S., and Inoue, K. (1993) Mammalian non-pancreatic phospholipases A_2. *Biochem. Biophys. Acta*, **117**, 217-231.

19. Gronich, J. H., Bonventre, J. V., and Nemenoff, R. A. (1990) Purification of a high-molecular-mass form of phospholipase A_2 from rat kidney activated at physiological calcium concentrations. *Biochem. J.*, **271**, 37-43.

20. Clark, J. D., Lin, L.-L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991) A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. *J. Biol. Chem.*, **65**, 1043-1051.

21. Takayama, K., Kudo, I., Kim, D. K., Nagata, K., Nozawa, T., and Inoue, K. (1991) Purification and characterization of human platelet phospholipase A_2 which preferentially hydrolyzes an arachidonoyl residue. *FEBS Letters*, **282**, 326-330.

22. Lin, L.-L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A., and Davis, R. J. (1993) cPLA$_2$ is phosphoryated and activated by MAP kinase. *Cell*, **72**, 269-278.

23. Hara, S., Chang, H. W., Horigome, K., Kudo, I., and Inoue, K. (1991) Purification of mammalian nonpancreatic extracellular phospholipase A2. *Methods Enzymol.*, **197**, 381-389.

24. Murakami, M., Kudo, I., and Inoue, K. (1993) Molecular nature of phospholipases A_2 involoved in prostaglandin I_2 synthesis in human umbilical vein endothelial cells. *J. Biol. Chem.*, **268**, 839-844.

25. Axelrod, J., Burch, R. J., and Jelsema, C. L. (1988) Receptor-mediated activation of phospholipase A_2 via GTP-binding proteins: arachidonic acid and its metabolites as second messengers. *Trends Neurosci.*, **11**, 117-123.

26. Exton, J. H. (1990) Signaling through phosphatidylcholine breakdown. *J. Biol. Chem.*, **265**, 1-4.

27. Habenicht, A. J. R., Salbach, P., Goerig, M., Zeh, W., Janssen-Timmen, U., Blattner, C., King, W. C., and Glomset, J. A. (1990) The LDL receptor pathway delivers arachidonic acid for eicosanoid formation in cells stimulated by platelet-derived growth factor. *Nature*, **345**, 634-636.

28. Habenicht, A. J. R., Dresel, H. A., Goerig, M., Weber, J., Stoehr, M., Glomset, J. A., Ross, R., and Schettler, G. (1986) Low density lipoprotein receptor-dependent prostaglandin synthesis in Swiss 3T3 cells stimulated by platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA*, **83**, 1344-1348.

29. Domin, J., and Rozengurt, E. (1993) Platelet-derived growth factor stimulates a biphasic mobilizaiton of arachidonic acid in Swiss 3T3 cells. *J. Biol. Chem.*, **268**, 8927-8934.

30. Habenicht, A. J. R., Goerig, M., Grulich, J., Rothe, D., Gronwald, R., Loth, U., Schettler, G., Kommerell, B., and Ross, R. (1985) Human platelet-derived growth factor stiumulates prostaglandin synthesis by activation and by rapid *de novo* synthesis of cyclooxygenase. *J. Clin. Invest.*, **75**, 1381-1387.

31. Murakami, M., Matsumoto, R., Austen, K. F., and Arm, J. P. (1994) Prostaglandin endoperoxide synthase-1 and -2 couple to different transmembrane stimuli to generate prostaglandin D_2 in mouse bone marrow-derived mast cells. *J. Biol. Chem.*, **269**, 22269-22275.

32. Reddy, S. T., and Herschman, H. R. (1994) Ligand-induced prostaglandin synthesis requires expression of the TIS10/PGS-2 prostaglandin synthase gene in murine fibroblasts and macrophages. *J. Biol. Chem.*, **269**, 15473-15480.

33. Smith, W. L., Marnett, L. J., and DeWitt, D. L. (1991) Prostaglandin and thromboxane biosynthesis. *J. Pharmac. Ther.*, **1991**, 153-179.

34. Tsai, A., Hsi, L. C., Kulmacz, R. J., Palmer, G., and Smith, W. L. (1994) Characterization of the tyrosyl radicals in ovine prostaglanidn H synthase by isotope replacement and site directed mutagenesis. *J. Biol. Chem.*, **269**, 5085-5091.

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

36. Picot, D., Loll, P.J., and Garavito, R.M. (1994) The X-ray srystal structure of the membrane protein prostalgandin H_2 synthase-1. *Nature*, **367**, 243-249.

37. Laneuville, O., Breuer, D. K., DeWitt, D. L., Hla, T., Funk, C., and Smith, W. L. (1994) Differential inhibiton of human prostaglandin endoperoxide H synthases-1 and -2 by nonsteroidal anti-inflammatory drugs. *J. Pharm. Exp. Therap.*, **271**, 927-934.

38. Kujubu, D. A., and Herschman, H. R. (1992) Dexamethasone inhibits mitogen induction of the TIS10 prostaglandin synthase/cyclooxygenase homologue. *J. Biol. Chem.*, **267**, 7991-7994.

39. Meade, E. A., Smith, W. L., and DeWitt, D. L. (1993) Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. *J. Biol. Chem.*, **268**, 6610-6614.

40. Percival, M. D., Ouellet, J., Vincent, C. J., Yergey, J. A., Kennedy, B. P., and O'Neill, G. P. (1994) Purification and characterization of recombinant human cyclooxygenase-2. *Arch. Biochem. Biophys.*, **315**, 111-118.

41. Meade, E. A. (1992) *Kinetic and Pharmacological Characterization of Murine PGH synthase-1 and PGH synthase-2*. Doctoral Dissertation, Department of Physiology, Michigan State University.

42. Rome, L. H., and Lands, W. E. M. (1975) Structural requirements of time-dependent inhibition of prostaglandin biosynthesis by anti-inflammatory drugs. *Proc. Natl. Acad. Sci. USA*, **72**, 4863-4865.

43. Meade, E. A., Smith, W. L., and DeWitt, D. L. (1993) Expression of murine prostaglandin (PGH) synthase-1 and PGH synthase-2 isozymes in *cos*-1 cells. *J. Lipid Mediators*, **6**, 119-129.

44. Mitchell, J. A., Akarasereemont, P., Thiemermann, C., Flower, R. J., and Vane, J. R. (1993) Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc. Natl. Acad. Sci. USA*, **90**, 11693-11697.

45. DeWitt, D. L., El-Harith, E. A., Kraemer, S. A., Andrews, M. J., Yao, E. F., Amstrong, R. L., and Smith, W. L. (1990) The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. *J. Biol. Chem.*, **265**, 5192-5198.

46. Lecomte, M., Laneuville, O., Ji, C., DeWitt, D. L., and Smith, W. L. (1994) Acetylation of human prostaglandin endoperoxide synthase-2 (cyclooxygenase-2) by aspirin. *J. Biol. Chem.*, **269**, 13207-13215.

47. Shimokawa, T., and Smith, W. L. (1992) Prostaglandin endoperoxide synthase, the aspirin acetylation region. *J. Biol. Chem.*, **267**, 12387-12392.

48. Masferrer, J. L., Zweifel, B. S., Manning, P. T., Hauser, S. D., Leahy, K. M., Smith, W. G., Isakson, P. C., and Seibert, K. (1994) Selective inhibition of inducible cyclooxygenase 2 *in vivo* is antiinflammatory and nonulcerogenic. *Proc. Natl. Acad. Sci. USA*, **91**, 3228-3232.

49. Seibert, K., Zhang, Y., Leahy, K., Hauser, S., Masferrer, J., Perkins, W., Lee, L., and Isakson, P. (1994) Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc. Natl. Acad. Sci. USA*, **91**, 12013-12017.

50. Bhattacharyya, D. K., Lecomte, M., Dunn, J., Morgans, D. J., and Smith, W. L. (1995) Selective inhibition of prostaglandin endoperoxide synthase-1 (cyclooxygenase-1) by valerylsalicylic acid. *Arch. Biochem. Biophys.*, **317**, 19-24.

51. Futaki, N., Takahashi, S., Yokoyama, M., Arai, S., Higuchi, S., and Otomo, S. (1994) NS-398, a new anit-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity in vitro. *Prostaglandins*, **47**, 55-59.

52. Hemler, M., Lands, W. E. M., and Smith, W. L. (1976) Purification of the cyclooxygenase that forms prostaglandins. Demonstration of two forms of iron in the holoenzyme. *J. Biol. Chem.*, **251**, 5575-5579.

53. van der Ouderaa, R. J., Buytnehek, M., Nugteren, D. H., and van Dorp, D. A. (1977) Purification and characterisation of prostaglandin endoperoxide synthetase from sheep vesicular glands. *Biochim. Biophys. Acta*, **487**, 315-331.

54. DeWitt, D. L., and Smith, W. L. (1988) Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence. *Proc. Natl. Acad. Sci. USA*, **85**, 1412-1416.

55. Merlie, J. P., Fagan, D., Mudd, J., and Needleman, P. (1988) Isolation and characterization of the complementary DNA for sheep seminal vesicle prostaglandin endoperoxide synthsae (cyclooxygenase). *J. Biol. Chem.*, **263**, 3550-3553.

56. Yokoyama, D., Takai, T., and Tanabe, T. (1988) Primary structure of sheep prostaglandin endoperoxide synthase deduced from cDNA sequence. *FEBS Lett.*, **231**, 347-351.

57. Xie, W., Chipman, J. G., Roberson, D. L., Erickson, R. L., and Simmons, D. L. (1991) Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci. USA*, **88**, 2692-2696.

58. Kujubu, D. A., Fletcher, B. S., Varnum, B. C., Lim, R. W., and Herschman, H. (1991) TIS10, a phorbol ester tumor promoter-inducible mRNA from swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J. Biol. Chem.*, **266**, 12866-12872.

59. O'Banion, M. K., Sadowski, H. B., Winn, V., and Young, D. A. (1991) A serum- and glucocorticoid-regulated 4-kilobase mRNA encodes a cyclooxygenase-related protein. *J. Biol. Chem.*, **266**, 23261-23267.

60. Funk, C. D., Funk, L. B., Kennedy, M. E., Pong, A. S., and Fitzgerald, G. A. (1991) Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. *FASEB Journal*, **5**, 2304-2312.

61. Hla, T., and Neilson, K. (1992) Human cyclooxygenase-2 cDNA. *Proc. Natl. Acad. Sci. USA*, **89**, 7384-7388.

62. O'Banion, M. K., Winn, V. D., and Young, D. A. (1992) cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. *Proc. Natl. Acad. Sci. USA*, **89**, 4888-4892.

63. Feng, L., Sun, W., Xia, Y., Tang, W. W., Chanmugan, P., Soyoola, E., Wilson, C. B., and Hwang, D. (1993) Cloning two isoforms of rat cyclooxygenase: differential regulation of their expression. *Arch. Biochem. Biophys.*, **307**, 361-368.

64. Sirois, J., and Richards, J. S. (1992) Purification and characterization of a novel, distinct isoform of prostaglandin endoperoxide synthase induced by human chorionic gonadotropin in granulosa cells of rat preovulatory follicles. *J. Biol. Chem.*, **267**, 6382-6388.

65. Herschman, H. R. (1994) Regulation of prostaglandin synthase-1 and prostaglandin synthase-2. *Cancer and Metastasism Reviews*, **13**, 241-256.

66. Kester, M., Coroneos, E., Thomas, P. J., and Dunn, M. J. (1994) Endothelin stimulates prostaglandin endoperoxide synthase-2 mRNA expression and protein synthesis through a tyrosine kinase-signaling pathway in rat mesangial cells. *J. Biol. Chem.*, **269**, 22574-22580.

67. Stroebel, M., and Goppelt-Stroebel, M. (1994) Signal transduction pathways responsible for serotonin-mediated prostaglandin G/H synthase expression in rat mesangial cells. *J. Biol. Chem.*, **269**, 22952-22957.

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

69. Evett, G. E., Zie, W., Chipman, J. G., Robertson, D. L., and Simmons, D. L. (1993) Prostaglandin G/H isoenzyme 2 expression in fibroblasts: regulation by dexamethasone, mitogens and oncogenes. *Arch. Biochem. Biophys.*, **306**, 169-177.

70. Kruijer, W., Cooper, J. A., Hunter, T., and Verma, I. M. (1984) Platelet-derived growth factor induces rapid and transient expression of the c-fos gene protein. *Nature*, **312**, 711-716.

71. Muller, R., Bravo, R., and Burckhardt, J. (1984) Induction of c-fos during myelomonocytic differentiation and macrophage proliferation. *Nature*, **314**, 546-548.

72. Kujubu, D. A., Reddy, S. T., Fletcher, B. S., and Herschman, H. R. (1993) Expression of the protein product of the prostaglandin synthase-2/TIS10 gene in mitogen-stimulated Swiss 3T3 cells. *J. Biol. Chem.*, **268**, 5425-5430.

73. Shaw, G., and Kamen, R. (1986) A conserved AU sequence from the 3'untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell*, **46**, 659-667.

74. Lee, S. H., Soyoola, E., Chanmugam, P., Hart, S., Zhong, H., Liou, S., Simmons, D., and Hwant, D. (1992) Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. *J. Biol. Chem.*, **267**, 25934-25938.

75. O'Sullivan, G. M., Chilton, F. H., Huggins, E. J., and McCall, C. E. (1992) Lipopolysaccharide induces prostaglandin H synthase-2 in aveolar macrophages. *Biochem. Biophys. Res. Commun.*, **187**, 1123-1127.

76. Ristimaki, A., Garfinkel, S., Wessendorf, J., Maciag, T., and Hla, T. (1994) Induction of cyclooxygenase-2 by interleukin-1α. *J. Biol. Chem.*, **269**, 11769-11775.

77. Crofford, L. J., Wilder, R. L., Ristimaki, A. P., Sano, J., Remmers, E. F., Epps, H. R., and Hla, T. (1994) Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. *J. Clin. Invest.*, **93**, 1095-1101.

78. Simmons, D. L., Zie, X., Chipman, J. G., and Evett, G. E. (1991) in *Prostaglandins, Leukotrienes, Lipoxins, and PAF* (Bailey, J. M., ed), pp. 67-78, New York.

79. O'Neill, G. P., and Ford-Hutchinson, A. W. (1993) Expression of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human tissues. *FEBS letters*, **330**, 156-160.

80. Smith, C. J., Morrow, J. D., Roberts II, L. J., and Marnett, L. J. (1993) Differentiation of monocytoid THP-1 cells with phorbol ester induces expression of prostaglandin endoperoxide synthase-1 (COX-1). *Biochem. Biophys. Res. Commun.*, **192**, 787-793.

81. Brannon, T. S., North, A. J., Wells, L. B., and Shaul, P. W. (1994) Prostacyclin synthesis in ovine pulmonary artery is developmentally regulated by changes in cyclooxygenase-1 gene expression. *J. Clin. Invest.*, **93**, 2230-2235.

82. Masferrer, J. L., Seibert, K., Zweifel, B., and Needleman, P. (1992) Endogenous glucocorticoids regulate an inducible cyclooxygenase enzyme. *Proc. Natl. Acad. Sci. USA*, **89**, 3917-3921.

83. DeWitt, D. L., Rollins, T. E., Day, J. S., Gauger, J. A., and Smith, W. L. (1981) Orientation of the active site and antigentic determinants of prostaglandin endoperoxide synthase in the endoplasmic reticulum. *J. Biol. Chem.*, **256**, 10375-10382.

84. Otto, J. C., DeWitt, D. L., and Smith, W. L. (1993) N-glycosylation of prostaglandin endoperoxide synthases-1and -2 and their orientations in the endoplamsic reticulum. *J. Biol. Chem.*, **268**, 18234-18242.

85. Mutsaers, G. H. G. M., Van Halbee, H., Kamerling, J. P., and Vliegenthart, J. F. G. (1985) Determination of the structure of the carbohydrate chains of prostaglandin endoperoxide synthase from sheep. *Eur. J. Biochem.*, **147**, 569-574.

86. Gebhart, A. M., and Ruddon, R. W. (1986) What regulates secretion of non-stored proteins by eukaryotic cells? *BioEssays*, **4**, 213-218.

87. Roth, G. J., Siok, C. J., and Ozols, J. (1980) Structural characteristics of prostaglandin synthetase from sheep vesicular gland. *J. Biol. Chem.*, **255**, 1301-1304.

88. Campbell, I. D., and Bork, P. (1993) *Curr. Opin. Struct. Biol.*, **3**, 385-392.

89. Otto, J. C., and Smith, W. L. (1994) The orientation of prostaglandin endoperoxide synthases-1 and -2 in the endoplasmic reticulum. *J. Biol. Chem.*, **269**, 19868-19875.

90. Smith, W. L., and Bell, T. G. (1978) Immunohistochemical localization of the prostaglandin-forming cyclooxygenase in renal cortex. *Am. J. Physiol.*, **235**, F451-F457.

91. Rollins, T. E., and Smith, W. L. (1980) Subcellular localization of prostaglandin-forming cyclooxygenase in Swiss mouse 3T3 fibroblasts by electron microscopic immunocytochemistry. *J. Biol. Chem.*, **255**, 4872-4875.

92. Regier, M. K., DeWitt, D. L., Schindler, M. S., and Smith, W. L. (1993) Subcellular localization of prostaglandin endoperoxide synthase-2 in murine 3T3 cells. *Arch. Biochem. Biophys.*, **301**, 439-444.

93. Morita, I., Schindler, M., Regier, M. K., Otto, J. C., DeWitt, D. L., and Smith, W. L. (1995) Different intracellular locations for prostaglandin endoperoxide H synthases-1 and -2. *J. Biol. Chem.*, in press

94. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D. (1994) *Molecular Biology of the Cell*, Third Ed.

95. Pante, N., and Aebi, U. (1993) The nuclear pore complex. *J. Cell Biol.*, **122**, 977-984.

96. Gerace, L. (1992) Molecular trafficking across the nuclear pore complex. *Curr. Opin. Cell Biol.*, **4**, 637-645.

97. Dingwell, C., and Laskey, R. A. (1991) Nuclear targeting sequences—a consensus? *TIBS*, **16**, 478-481.

98. Kalderon, D., Roberts, B. L., Richardson, W. D., and Smith, A. E. (1984) A short amino acid sequence able to specify nuclear location. *Cell*, **39**, 499-509.

99. von Heijne, G. (1985) Signal Sequences. The limits of variation. *J. Mol. Biol.*, **184**, 99-105.

100. Bird, P., Gething, M. J., and Sambrook, J. (1990) The functional efficiency of a mammalian signal peptide is directly related to its hydrophobicity. *J. Biol. Chem.*, **265**, 8420-8425.

101. Sanders, S. L., and Schekman, R. (1992) Polypeptide translocation across the endoplasmic reticulum membrane. *J. Biol. Chem.*, **267**, 13791-13794.

102. Gilmore, R. (1991) The protein translocation apparatus of the rough endoplasmic reticulum, its associated proteins, and the mechansim of translocation. *Curr. Opin. Cell Biol.*, **3**, 580-584.

103. Simon, S. (1993) Translocation of proteins across the endoplamsic reticulum. *Curr. Opin. Cell Biol.*, **5**, 581-588.

104. Walter, P., and Lingappa, V. R. (1986) Mechanism of protein translocation across the endoplamsic reticulum membrane. *Annu. Rev. Cell Biol.*, **2**, 499-516.

105. Rapport, T. A. (1991) Protein transport across the endoplasmic reticulum membrane: facts, models, mysteries. *FASEB Journal*, **5**, 2792-2798.

106. Singer, S. J. (1990) The structure and insertion of integral proteins in membranes. *Annu. Rev. Cell Biol.*, **6**, 247-296.

107. High, S., and Dobberstein, B. (1992) Mechanisms that determine the transmembrane disposition of proteins. *Curr. Opin. Cell Biol.*, **4**, 581-586.

108. Stuart, R. A., Cyr, D. M., Craig, E. A., and Neupert, W. (1994) Mitochondrial molecular chaperones: their role in protein translocation. *TIBS*, **19**, 87-92.

109. Lithgow, T., Blick, B. S., and Schatz, G. (1995) The protein import receptor of mitochondria. *TIBS*, **20**, 98-101.

110. Rothman, J. E. (1987) Protein sorting by selective retention in the endoplasmic reticulum and golgi stack. *Cell*, **50**, 521-522.

111. Burgess, T. L., and Kelly, R. B. (1987) *Annu. Rev. Cell Biol.*, **3**, 243-293.

112. Rothman, J. E., and Orci, L. (1992) Molecular dissection of the secretory pathway. *Nature*, **355**, 409-415.

113. Dahms, N. M., Lobel, P., and Kornfeld, S. (1989) Mannose 6-phosphate receptors and lysosomal enzyme targeting. *J. Biol. Chem.*, **264**, 12115-12118.

114. Pelham, H. R. B., Hardwick, K. G., and Lewis, M. J. (1988) Sorting of soluble ER proteins in yeast. *EMBO J.*, **7**, 1757-1762.

115. Bennett, M. K., and Scheller, R. H. (1993) The molecular machinery for secretion is conserved from yeast to neurons. *Proc. Natl. Acad. Sci. USA*, **90**, 2559-2563.

116. Rothman, J. E. (1994) Mechanisms of intracellular protein transport. *Nature*, **372**, 55-63.

117. Luzio, J. P., and Banting, G. (1993) Eukaryotic membrane traffic: retrieval and retention mechanisms to achieve organelle residence. *TIBS*, **18**, 395-398.

118. Blobel, G. (1980) Intracellular protein topogenesis. *Proc. Natl. Acad. Sci. USA*, **77**, 1496-1500.

119. Pelham, H. R. B. (1990) The retention signal for soluble proteins of the endoplasmic reticulum. *TIBS*, **15**, 483-485.

120. Nilsson, T., Jackson, M. R., and Peterson, P. A. (1989) Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell*, **48**, 707-718.

121. Pelham, H. R. B. (1989) Control of protein exit from the endoplasmic reticulum. *Annu. Rev. Cell Biol.*, **5**, 1-23.

122. Sweet, D. J., and Pelham, H. R. B. (1992) The *Saccharomyces cerevisiae* SEC20 gene encodes a membrane glycoprotein which is sorted by the HDEL retrieval system. *EMBO J.*, **11**, 423-432.

123. Munro, S., and Pelham, H. R. B. (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell*, **48**, 899-907.

124. Denecke, J., De Rycke, R., and Botterman, J. (1992) Plant and mammalian sorting signals for protein retention in the endoplasmic reticulum contain a conserved epitope. *EMBO J.*, **11**, 2345-2355.

125. Zagouras, P., and Rose, J. K. (1989) Carboxy-terminal SEKDEL sequences retard but do not retain two secretory proteins in the endoplasmic reticulum. *J. Cell Biol.*, **109**, 2633-2640.

126. Mazzarella, R. A., Marcus, N., Haugejorden, S. M., Balcarek, J. M., Baldassare, J. J., Roy, B., Li, L.-j., Lee, A. S., and Green, M. (1994) ERp61 is GRP58, a stress-inducible luminal endoplasmic reticulum protein, but is devoid of phosphatidylinositide-specific phospholipase C activity. *Arch. Biochem. Biophys.*, **308**, 454-460.

127. Andres, D. A., Dickerson, I. M., and Dixon, J. E. (1990) Variants of the carboxyl-terminal KDEL sequence direct intracellular retention. *J. Biol. Chem.*, **265**, 5952-5955.

128. Robbi, M., and Beaufay, H. (1991) The COOH terminus of several liver carboxylesterases targets these enzymes to the lumen of the endoplasmic reticulum. *J. Biol. Chem.*, **266**, 20498-20503.

129. Munro, S., and Pelham, H. R. B. (1986) An hsp70-like protein in the ER: identity with glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell*, **46**, 291-300.

130. Sorger, R. K., and Pelham, H. R. B. (1987) The glucose regulated protein grp94 is related to heat shock protein hsp90. *J. Mol. Biol.*, **194**, 341-344.

131. Tang, B. L., Wong, S. H., Low, S. H., and Hong, W. (1992) Retention of a type II surface membrane protein in the endoplasmic reticulum by the Lys-Asp-Glu-Leu sequence. *J. Biol. Chem.*, **267**, 7072-7076.

132. Ceriotti, A., and Colman, A. (1988) Binding to membrane proteins within the endoplasmic reticulum cannot eplain the retention of the glucose-regulated protein GRP78 in *Xenopus* oocytes. *EMBO J.*, **7**, 633-638.

133. Pelham, H. R. B. (1988) Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO J.*, **7**, 913-918.

134. Dean, N., and Pelham, H. R. B. (1990) Recycling of proteins from the Golgi compartment to the ER in yeast. *J. Cell Biol.*, **111**, 369-377.

135. Semenza, J. C., Hardwick, K. B., Dean, N., and Pelham, H. R. B. (1990) ERD2, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell*, **61**, 1349-1357.

136. Lewis, M. J., and Pelham, H. R. B. (1990) A human homologue of the yeast HDEL receptor. *Nature*, **348**, 162-163.

137. Lewis, M. J., and Pelham, H. R. B. (1992) Sequence of a second human KDEL receptor. *J. Mol. Biol.*, **226**, 9130-916.

138. Tang, B. L., Wong, H. S., Qi, X. L., Low, S. H., and Hong, W. (1993) Molecular cloning, characterization, subcellular localization and dynamics of p23, the mammalian KDEL receptor. *J. Cell Biol.*, **120**, 325-338.

139. Townsley, F. M., Wilson, D. W., and Pelham, H. R. B. (1993) Mutational analysis of the human KDEL receptor: distinct structural requirements for Golgi retention, ligand binding and retrograde transport. *EMBO J.*, **12**, 2821-2829.

140. Wilson, D., Lewis, M. J., and Pelham, H. R. B. (1993) pH dependent binding of KDEL to its receptor *in vitro*. *J. Biol. Chem.*, **268**, 7465-7468.

141. Jackson, M. R., Nilsson, T., and Peterson, P. A. (1990) Identification of a consensus motif for retention of transmembrane proteins in the endoplamsmic reticulum. *EMBO J.*, **9**, 3153-3162.

142. Shin, J., Dunbrack, R. L., Lee, S., and Strominger, J. L. (1991) Signals for retention of transmembrane proteins in the endoplamsic reticulum studies with CD4 truncation mutants. *Proc. Natl. Acad. Sci. USA*, **88**, 1918-1922.

143. Gaynor, E. C., te Heesen, S., Graham, T. R., Aebi, M., and Emr, S. D. (1994) Signal-mediated retrieval of a membrane protein from the golgi to the ER in yeast. *J. Cell Biol.*, **127**, 653-665.

144. Lippincott-Schartz, J., Donaldson, J. G., Schweizer, A., Berger, E. G., Hauri, H.-P., Yuan, L. C., and Klausner, R. D. (1990) Microtuble-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. *Cell*, **60**, 821-836.

145. Jackson, M. R., Nilsson, T., and Peterson, P. A. (1993) Retrieval of transmembrane proteins to the endoplasmic reticulum. *J. Cell Biol.*, **121**, 317-333.

146. Dingwall, C., and Laskey, R. (1992) The nuclear membrane. *Science*, **258**, 942-947.

147. Wozniak, R. W., and Blobel, G. (1992) The single transmembrane segment of gp210 is sufficient for sorting to the pore membrane domain of the nuclear envelope. *J. Cell Biol.*, **119**, 1441-1449.

148. Ye, Q., and Worman, H. (1994) Primary structure analysis and lamin B and DNA binding of human LBR, and integral protein of the nuclear envelope inner membrane. *J. Biol. Chem.*, **269**, 11306-11311.

149. Soullman, B., and Worman, H. J. (1993) The amino-terminal domain of the lamin B receptor is a nuclear envelope targeting signal. *J. Cell Biol.*, **120**, 1093-1100.

150. Kumar, N., Syin, C., Carter, R., Quakyi, I., and Miller, L. H. (1988) *Plasmodium falciparum* gene encoding a protein similar to the 78-kDa rat glucose-regulated stress protein. *Proc. Natl. Acad. Sci. USA*, **85**, 6277-6281.

151. Sanger, F. S., Nicklen, S., and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467.

152. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.*, **154**, 367-382.

153. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed.

154. Luthman, H., and Magnusson, G. (1983) High efficiency polyoma DNA transfection of chloroquine treated cells. *Nucleic Acids Res.*, **11**, 1295-13008.

155. Laemmli, U. K. (1970) Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-687.

156. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electorphoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*, **76**

157. Marnett, L. J., Chen, Y. N., Maddipati, K. R., Ple, P., and Labeque, R. (1989) Functional differentiation of cyclooxygenase and peroxidase activities of prostaglandin synthase by trypsin treatment. *J. Biol. Chem.*, **263**, 16532-16535.

158. DeWitt, D. L., Day, J. S., Gauger, J. A., and Smith, W. L. (1982) Monoclonal antibodies against PGH synthase: an immunoradiometric assay for quantitating the enzyme. *Methods Enzymol.*, **86**, 229-240.

159. Diaz, A., Reginato, A. M., and Jimenez, S. A. (1992) Alternative splicing of human prostaglandin G/H synthase mRNA and evidence of differential regulation of the resulting transcripts by transforming growth factor β1, interleukin 1β and tumor necrosis factor α. *J. Biol. Chem.*, **267**, 10816-10822.

160. Vane, J. R. (1976) in *Advances in Prostaglandin and Thromboxane Research* (Samuelsson, B., and Paloetti, R., eds) Vol. 2, pp. 791-802, Raven Press, New York.

161. Davies, P., Bailey, P. J., Goldenburg, M. M., and Ford-Hutchinson, A. W. (1984) in *Annu. Rev. Immunol.*, **2**, 335-357.

162. Smith, W. L., Sonnenburg, W. K., Allen, M. L., Watanabe, T., Zuh, J., and El-Harith, E. A. (1989) in *Renal Eicosanoids* (Dunn, M. J., Patrono, C., and Cinotti, G. A., eds), pp. 131-147, Plenum, New York.

163. Espey, L. L., Norris, C., and Saphire, D. (1986) *Endocrinology*, **119**, 746-756.

164. Tsafrir, A. A., Koch, Y., and Lindner, H. R. (1973) Ovulation rate and serum LH levels in rats treated with indomethacin of prostaglandin E_2. *Prostaglandins*, **3**, 461-470.

165. LeMaire, W. J., Clark, M. R., and Marsh, J. M. (1978) in *Human Ovulation: Mechanism, Detection and Regulation* (Hafez, E. S. E., ed), pp. 159-175, Elsevier, North Holland, Amsterdam.

166. Armato, U., and Andreis, P. G. (1983) Prostaglandins in the F series are extremely powerful growth factors for primary neonatal rat hepatocytes. *Life Science*, **33**, 1745-1755.

167. Jimenez de Asua, L., Richmond, K. M., and Otto, A. M. (1981) Two growth factors and two hormones regulate initiation of DNA synthesis in cultured mouse cells through different pathways of events. *Proc. Natl. Acad. Sci. USA*, **78**, 1004-1008.

168. Kulmacz, R. J., and Lands, W. E. M. (1984) Prostaglandin H synthase: stoichiometry of heme cofactor. *J. Biol. Chem.*, **259**, 6358-6363.

169. Smith, W. L., and Marnett, L. J. (1991) Prostaglandin endoperoxide synthase: structure and catalysis. *Biochem. Biophys. Acta*, **1093**, 1-17.

170. Smith, W. L., and Wilkin, G. P. (1977) Immunohiotochemistry of prostaglandin endoperoxide-forming cyclooxygenases. *Prostaglandins*, **13**, 873-892.

171. Smith, W. L., DeWitt, D. L., and Allen, M. L. (1983) Bimodal distribution of the Prostaglandin I_2 synthase antigen in smooth muscle cells. *J. Biol. Chem.*, **258**, 5922-5926.

172. Weller, P. F., Ryeom, S. W., and Dvorak, A. M. (1991) in *Prostaglandins, Leukotrienes, Lipoxins, and PAF* (Bailey, J. M., ed), pp. 353-362, Plenum, New York.

173. Ryseck, R. P., Raynoschek, D., Macdonald-Bravo, H., Dorfan, K., Mattei, M. G., and Bravo, R. (1992) Identification of an immediate early gene pghs-B whose protein product has prostaglandin synthase/cyclooxygenase activity. *Cell Growth and Differentiation*, **3**, 443-450.

174. Fletcher, B. S., Kujubu, D. A., Perrin, D. M., and Herschman, H. R. (1992) Structure of the mitogen-inducible TIS10 gene and demonstration that the TIS10-encoded protein is a functional prostaglandin synthase. *J. Biol. Chem.*, **267**, 4338-4344.

175. Mumby, S., Pang, I., Gilman, A. G., and Sternweis, P. C. (1988) Chromatographic resolution and immunologic identification of the $\alpha 40$ and $\alpha 41$ subunits of guanine nucleotide-binding regulatroy proteins from bovine brain. *J. Biol. Chem.*, **263**, 2020-2026.

176. Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orci, L., and Klausner, R. D. (1991) Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. *Cell*, **67**, 601-616.

177. Teraksaki, M., and Reese, T. S. (1992) Characterization of endoplasmic reticulum by colocalization of BiP and dicarbocyanine dyes. *J. Cell Sci.*, **101**, 315-322.

178. Willingham, M. D., and Pastan, I. (1985) *An Atlas of Immunofluorescence in Cultured Cells*, Academic Press, New York.

179. Regier, M. K., Otto, J. C., DeWitt, D. L., and Smith, W. L. (1995) Localization of prostaglandin endoperoxide synthase-1 to the endoplasmic reticulum and nuclear envelope is independent of its C-terminal tetrapeptide -PTEL. *Arch. Biochem. Biophys.*, **317**, 457-463.

180. Capecchi, M. R. (1980) High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell*, **22**, 479-488.

181. Wade, M. H., DeFeijter, A. W., Frame, M. K., and Schindler, M. (1993) Quantitative fluorescence imaging techniques for the study of organization and signaling mechanisms in cells. *Bioanalytical Instrumentation*, **37**, 117-141.

182. Lin, L.-L., Lin, A. Y., and DeWitt, D. L. (1992) IL-1α induces the accumulation of $cPLA_2$ and the release of PGE_2 in human fibroblasts. *J. Biol. Chem.*, **267**, 23451-23454.

183. Schalkwijk, C. G., Vervoordeldonk, M., Pfeilschifter, J., and van den Bosch, H. (1993) Interleukin-1β-induced cytosolic phospholipase A_2 activity and protein synthesis is blocked by dexamethasone in rat mesangial cells. *FEBS Letters*, **333**, 339-343.

184. Nakamura, T., Lin, L.-L., Kharbanda, S., Knopf, J., and Kufe, D. (1992) Macrophage colony stimulating factor activates phosphatidylcholine hydrolysis by cytoplasmic phospholipase A_2. *EMBO J.*, **11**, 4917-4922.

185. Lin, L.-L., Lin, A. Y., and Knopf, J. L. (1992) Cytosolic phospholipase A_2 is coupled to hormonally regulated release of arachidonic acid. *Proc. Natl. Acad. Sci. USA*, **89**, 6147-6151.

186. Kramer, R. M., Roberts, E. F., Manetta, J. V., Hyslop, P. A., and Jakubowski, J. A. (1993) Thrombin-induced phosphorylation and activation of Ca^{2+}-sensitive cytosolic phospholipase A_2 in human platelets. *J. Biol. Chem.*, **268**, 26796-26804.

187. Kast, R., Furstenberger, G., and Marks, F. (1993) Activation of cytosolic phospholipase A_2 by transforming growth factor-α in HEL-30 keratinocytes. *J. Biol. Chem.*, **268**, 16795-16802.

188. Chepenik, K. P., Diaz, A., and Jimenez, S. A. (1994) Epidermal growth factor coordinately regulates the expression of prostaglandin G/H synthase and cytosolic phopholipase A_2 genes in embryonic mouse cells. *J. Biol. Chem.*, **269**, 21786-21792.

189. Sa, G., Murugesan, G., Jaye, M., Ivashchenko, Y., and Fox, P. L. (1995) Activation of cytosolic phospholipase A_2 by basic fibroblast growth factor via a p42 mitogen-activated protein kinase-dependent phosphorylation pathway in endothelial cells. *J. Biol. Chem.*, **270**, 2360-2366.

190. Barnett, R. L., Ruffini, L., Hart, D., Mancuso, P., and Nord, E. P. (1994) Mechanism of endothelin activation of phospholipase A_2 in rat renal medullary interstitial cells. *Am. J. Physiol.*, **266**, F46-F56.

191. Hanahan, D. J. (1986) Platelet activating factor: a biologically active phosphoglyceride. *Annu. Rev. Biochem.*, **55**, 483-509.

192. Ramesha, C. S., and Ives, D. L. (1993) Detection of arachidonoyl-selective phospholipase A_2 in human neutrophil cytosol. *Biochem. Biophys. Acta*, **1168**, 37-44.

193. Hulkower, K. I., Hope, W. C., Chen, T., Anderson, C. M., Coffey, J. W., and Morgan, D. W. (1992) Interleukin-1β stimulates cytosolic phospholipase A_2 in rheumatoid synovial fibroblasts. *Biochem. Biophys. Res. Commun.*, **184**, 712-718.

194. Sharp, J. D., and White, D. L. (1993) Cytosolic PLA_2: mRNA levels and potential for transcriptional regulation. *J. Lipid Mediators*, **8**, 183-189.

195. Hayakawa, M., Ishida, N., Takeuchi, K., Shibamoto, S., Hori, T., Oku, N., Ito, F., and Tsujimoto, M. (1993) Arachidonic acid-selective cytosolic phospholipase A_2 is crucial in the cytotoxic action of tumor necrosis factor. *J. Biol. Chem.*, **268**, 11290-11295.

196. Gil, J., Higgins, T., and Rozengurt, E. (1991) Mastoparan, a novel mitogen for Swiss 3T3 cells, stimulates pertussis toxin-sensitive arachidonic acid release without inositol phosphate accumulation. *J. Cell Biol.*, **113**, 943-950.

197. Nemenoff, R. A., Winitz, S., Qian, N.-X., Van Putten, V., Johnson, G. L., and Heasley, L. E. (1993) 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.*, **268**, 1960-1964.

198. Channon, J. Y., and Leslie, C. C. (1990) 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.*, **265**, 5409-5413.

199. Durstin, M., Durstin, S., Molski, T. F. P., Becker, E. L., and Sha'afi, R. I. (1994) Cytoplasmic phospholipase A_2 translocates to membrane fraction in human neutrophils activated by stimuli that phosphorylate mitogen-activated protein kinase. *Proc. Natl. Acad. Sci. USA*, **91**, 3142-3146.

200. Nalefski, E. A., Sultzman, L. A., Martin, D. M., Kriz, R. W., Towler, P. S., Knopf, J. L., and Clark, J. D. (1994) Delineation of two functionally distinct domains of cytosolic phospholipase A_2, a regulatory Ca^{2+}-dependent lipid-binding domain and a Ca^{2+}-independent catalytic domain. *J. Biol. Chem.*, **269**, 18239-18249.

201. Peters-Golden, M., and McNish, R. W. (1993) Redistribution of 5-lipoxygenase and cytosolic phospholipase A_2 to the nuclear fraction upon macrophage activation. *Biochem. Biophys. Res. Commun.*, **196**, 147-153.

202. Woods, J. W., Evans, J. F., Ethier, D., Scott, S., Vickers, P. J., Hearn, L., Heibein, J. A., Charleson, S., and Singer, I. I. (1993) 5-lipoxgenase and 5-lipoxygenase-activating protein are localized in the nuclear envelope of activated human leukocytes. *J. Exp. Med.*, **178**, 1935-1946.

203. Brock, T. G., Paine, R. I., and Peters-Golden, M. (1994) Localization of 5-lipoxygenase to the nucleus of unstimulated rat basophilic leukemia cells. *J. Biol. Chem.*, **269**, 22059-22066.

204. Larkin, M., Ahern, T. J., Stoll, M. S., Shaffer, M., Sako, D., Opbrien, J., Yuen, C.-T., Lawson, A. M., Childes, R. A., Barone, K. M., Langer-Safer, P. R., Hasegawa, A., Kiso, M., Larsen, G. R., and Feizi, T. (1992) Spectrum of sialylated and nonsialylated fuco-oligosaccharides bound by the enodthelial-leukocyte adhesion molecule E-selectin. *J. Biol. Chem.*, **267**, 13661-13668.

205. Capriotti, A. M., Furth, E. E., Arrasmith, M. E., and Laposata, M. (1988) Arachidonate released upon agonist stimulation preferentially originates from arachidonate most recently incorporated into nuclear membrane phospholipids. *J. Biol. Chem.*, **263**, 10029-10034.

206. Neufeld, E. J., Majerus, P. W., Krueger, C. M., and Saffitz, J. E. (1985) Uptake and subcellular distribution of [^{3}H]arachidonic acid in murine fibrosarcoma cells measured by electron microscope autoradiography. *J. Cell Biol.*, **101**, 573-581.

207. Roshak, A., Sathe, G., and Marshall, L. A. (1994) Suppression of monocyte 85-kDa phospholipase A2 by antisense and effect on endotoxin-induced prostaglandin biosynthesis. *J. Biol. Chem.*, **269**, 25999-26005.

208. Balsinde, J., Barbour, S. E., Bianco, I. D., and Dennis, E. A. (1994) Arachidonic acid moilization in P388D1 macrophages is controlled by two distinct Ca^{2+}-dependent phopholipase A2 enzymes. *Proc. Natl. Acad. Sci. USA*, **91**, 11060-11064.

209. Appleby, S. B., Ristimaki, A., Neilson, K., Narko, K., and Hla, T. (1994) Structure of the human cyclo-oxygenase-2 gene. *Biochem. J.*, **302**, 723-727.