LOCALIZATION OF HUMAN PROSTAGLANDIN E2 RECEPTORS IN POLARIZED EPITHELIAL CELLS

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ABSTRACT

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The underlying mechanisms of protein sorting in polarized epithelial cells are poorly understood. Several studies have determined membrane targeting of G protein-coupled receptors (GPCRs) using epithelial cells such as Madin-Darby canine kidney (MDCK) cells. Polarized epithelial cells are composed of apical and basolateral plasma membrane domains with specific protein compositions separated by tight junctions. Purinergic, muscarinic, and adrenergic receptors are a few examples of GPCRs that have been shown to localize to specific membranes in MDCK cells. The current work seeks to determine the differences in subcellular localization of the human prostaglandin E_2 receptors. The EP receptors are all GPCRs, which differ in their second messenger pathways. The EP3 receptor is unique in that it has eight different isoforms that differ in the lengths of the carboxyl tail.

The EP3 isoforms, as well as the EP2 and EP4 receptors, have distinct properties, including different agonist-induced internalization patterns. We have also shown the EP3 isoforms have tissue-specific distribution patterns.

To further study the differences among the PGE_2 receptors, we examined their subcellular localization patterns in polarized epithelial cells. We have determined the unique subcellular localization patterns for the receptors, as well as three mutants in MDCK cells. The localization patterns for these receptors in human bronchial epithelial (BEAS-2B) cells were quite similar to the MDCK cells, suggesting that receptor localization is not dependent on cell type. Additionally, in an attempt to locate structural motifs responsible for apical or basolateral localization, receptor chimeras between the purinergic $P2Y_2/P2Y_4$ receptors and EP3.VI isoform were constructed. Overall, the aim of our work was to define the subcellular localization patterns of various human prostaglandin E_2 receptors and our results suggest the differences among them may correlate to the diverse physiological actions of PGE_2 throughout the body.

DEDICATION

This dissertation is dedicated to my family and friends, especially…

To my parents, Pete and Monica Albano. I can't thank you both enough for your unconditional love and support. You have provided an environment that gave me the confidence to pursue my goals and even when I was consumed with self-doubt, your belief in me never wavered. Because of you, I know that with hard work, and a little bit of luck, all the puzzle pieces will fall into place.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Overview

The sorting of proteins to specific cellular locations is important for proper functioning of these proteins; however, the underlying mechanisms of protein sorting are poorly understood. Several studies have determined membrane targeting of G protein-coupled receptors using polarized epithelial cells such as Madin-Darby canine kidney (MDCK) cells. Polarized epithelial cells are composed of apical and basolateral plasma membrane domains with specific protein compositions separated by tight junctions (Rodriguez-Boulan et al., 1989). Purinergic, muscarinic, and adrenergic receptors are a few examples of GPCRs that have been shown to localize to specific membranes in MDCK cells. The current work seeks to determine the differences in subcellular localization of several of the human prostaglandin E_2 receptors.

Prostaglandin E_2 (PGE_2) is an endogenous autacoid derived from arachidonic acid via the cyclooxygenase pathway. It is involved in a variety of processes throughout the human body, such as the inhibition of gastric secretion, dilation and constriction of blood vessels, and the induction of fever in response to infection or inflammation (Coleman et al., 1994; Ushikubi et al., 1998). The effects of PGE_2 are mediated by binding to four distinct receptors, designated EP1, EP2, EP3, and EP4. The EP receptors are all G protein-coupled receptors (GPCRs) that show similar affinity for PGE_2; however, they differ in their second messenger pathways (for review see Coleman et al., 1994). The human EP3 receptor is unique in that it exists as eight different isoforms: EP3.I, EP3.II, EP3.III, EP3.IV, EP3.V, EP3.VI, EP3.e and EP3.f.

These isoforms are identical in sequence through the first 359 amino acids but differ in the length and composition of their carboxyl-tails, ranging from 6 to 66 amino acids in length (Kotani et al., 1997). While all the isoforms inhibit adenylyl cyclase via the G_i pathway, they have also been shown to couple to G_s, G_q and $G_{12/13}$, suggesting the isoforms may be responsible for a wide variety of physiological functions (An et al., 1994). The human EP3 isoforms have also been shown to differ in other properties, including constitutive activity (Jin et al., 1997).

Previous studies in our laboratory determined that the EP3 isoforms display different agonist-induced internalization patterns in HEK293 cells (Bilson et al., 2004). Further examination revealed that the isoforms also differ in their internalization pathways. EP3.I and EP3.f receptors internalize by a clathrin-dependent pathway, while EP3.II receptors undergo clathrin-independent internalization. Mutation studies performed in our laboratory also revealed a five amino acid motif, SSSTS, located at amino acid 369 within the C-tail of the EP3.I receptor played a critical role in the internalization of this receptor. Mutation of the serine and threonine residues in this sequence to alanine completely blocked internalization of the receptor.

Our laboratory also studied the internalization of the EP2 and EP4 receptors in HEK293 cells. It was shown that upon agonist stimulation with PGE_2, the EP4 receptor internalized completely while the EP2 receptor did not internalize (Desai et al., 2000). In this work, it was also shown that the C-tail of the EP4 receptor played a critical role in receptor internalization; however, serine and threonine residues were not involved (Desai et al., 2000).

Our current studies investigated the subcellular localization patterns of the PGE_2 receptors in Madin-Darby canine kidney (MDCK) and human bronchial epithelial (BEAS-2B) cells. We have determined the unique subcellular localization patterns for the receptors, as well as three mutants in MDCK cells. The localization patterns for these receptors in BEAS-2B cells were quite similar to the MDCK cells, suggesting that receptor localization is not dependent on cell type.

We have also constructed various receptor chimeras to locate structural motifs responsible for apical or basolateral localization. We showed that the C-tails of the EP3 isoforms play a significant role in determining the subcellular localization for those receptors. We created chimeras of the basolaterally localized EP3.VI receptor with the purinergic $P2Y_2$ and $P2Y_4$ receptors, which localized apically. We hypothesized that the known sorting signals within each chimera would dictate the subcellular localization for these constructs and attempted to demonstrate this in MDCK cells.

Overall our work defines the subcellular localization patterns of various human prostaglandin E_2 receptors and suggests the differences among them may correlate to the diverse physiological actions of PGE_2 throughout the body.

Epithelial Cell Composition and Protein Sorting

Epithelial cells are found throughout the body and serve as a dynamic barrier between internal and external environments. These cells are arranged as sheets, tightly connected to one another. There are three types of epithelium: squamous, columnar, and cuboidal. These types of tissues are arranged as simple (a single layer) or stratified

(many cells in thickness). In the human body, epithelial cells line the gastrointestinal tract, lungs, reproductive and urinary tracts. They also line the mucous membranes and can be found on the surface of the cornea and the kidney.

Structurally, epithelial cells are highly polarized, displaying distinct apical and basolateral plasma membrane domains, separated by tight junctions **(Figure 1)** (Rodriguez-Boulan et al., 1989). Tight junctions, or zonula occludens, function to connect epithelial cells to one another, preventing the diffusion of solutes and fluids around the cells. Additionally, they define the boundary between the apical and basolateral surface of the plasma membrane. The apical and basolateral domains are composed of specific proteins and lipids, allowing each surface to serve distinct functions. The apical domain of the plasma membrane faces the outside of a compartment or organ. It is also referred to as the luminal surface. In some tissues, microvilli project out from the apical domain to increase surface area for the transport and enzymatic activities at this surface. The basolateral domain of epithelial cells faces adjacent cells and the extracellular fluid compartment. It indirectly comes in contact with the blood supply to transport water and ions. The basolateral surface is connected to the basal lamina, or basement membrane, which functions to structurally support the epithelial tissue.

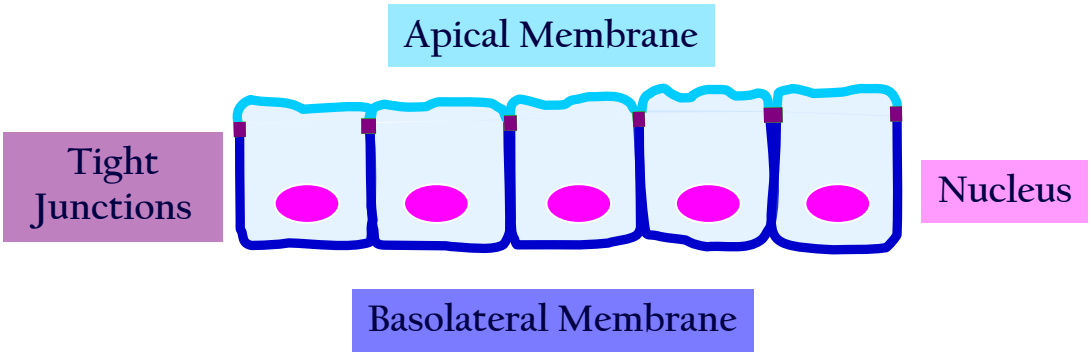


Figure 1. Epithelial Cell Structure

Epithelial cells are composed of an apical surface, which faces the lumen, and a basolateral surface, which faces adjacent cells and the extracellular fluid compartment. Each surface has a unique lipid and protein composition and they are separated from one another by tight junctions, or zonula occludens.

The specific proteins that are located at a particular surface are directed there via two methods. Newly synthesized proteins can be sorted directly to a specific surface via transport vesicles. Proteins can also be sorted via an indirect pathway. In this route, proteins are sorted first to one surface (usually the basolateral surface) and they are endocytosed into a vesicle which then carries them to the apical surface. Madin-Darby canine kidney cells, which are commonly used in protein sorting studies and are used in this thesis, primarily use the direct pathway to traffic apically targeted proteins (Mostov et al., 2000).

Sorting of G Protein-Coupled Receptors

G protein-coupled receptors (GPCRs) are integral membrane proteins that control various physiological and behavioral signaling pathways. Functional changes to the

receptor, including agonist interactions and trafficking to various cellular compartments, allow these receptors to play a very dynamic role. One key component of GPCR function is the localization of the receptor at a specific membrane site. Cell surface localization is governed by two principle mechanisms: trafficking of the receptor to a particular site and retention of the receptor at that site (Tan et al., 2004). The functional significance of GPCR localization is demonstrated by various diseases that arise from the mislocalization of receptors, such as retinitis pigmentosa (Humphries et al., 1992), X-linked nephrogenic diabetes insipidus (Oksche and Rosenthal, 1998), and hypogonadotropic hypogonadism (Layman et al., 1998).

In the current literature, studies show that many GPCRs display distinct subcellular localization patterns in polarized epithelial cells **(Table 1)**. These patterns play a critical role in allowing GPCRs to mediate the appropriate responses to hormones, neurotransmitters, local mediators and other endogenous compounds. As there is a significant amount of information on GPCR localization, only a few examples will be provided in this thesis, including purinergic, muscarinic, adenosine and adrenergic receptors.

Table 1. GPCR Localization Studies in MDCK Cells

Receptor	Reference
P2Y Nucleotide	Zambon et al., 2001; Wolff et al., 2004
Muscarinic Acetylcholine	Simon and Baggett, 1992; Toescu et al., 1992; Rouse and Levey, 1996; Wess et al., 1996; Nadler et al., 1999; Nadler et al., 2000; Iverson et al., 2005
Alpha 2-Adrenergic	Keefer and Limbird, 1993; Saunders et al., 1995: Wozniak and Limbird, 1995; Saunders et al., 1998; Olli-Lahdesmaki et al., 1999; Tan et al., 2004
A1 Adenosine	Saunders et al., 1995; Casavola et al., 1996; Saunders et al., 1996
Glutamate	Matsuda and Mishina, 2000; McCarthey et al., 2001; Cheng et al., 2002
Thyrotropin-Stimulating Hormone	Beau et al., 2004

Nucleotide binding P2Y receptors serve an important role in ion and nutrient transport in epithelial cells and many cells express multiple subtypes of P2Y receptors (Post et al., 1996; Insel et al., 2001; Leipzig, 2003). This receptor family is comprised of eight subtypes: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$. These receptor subtypes are distinguished by their second messenger pathways. $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ couple to G_q, while $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ couple to $G_{i/o}$. P2Y receptors have been shown to localize to distinct membrane surfaces in polarized epithelial cells. Wolff et al. (2004) showed that all but one of the eight P2Y receptors localized exclusively to either the apical or basolateral membrane surfaces of MDCK cells. A similar targeting profile was obtained in lung 16HBE14o- and colonic Caco-2

cells, suggesting that targeting of P2Y receptors is not a function of the cell line in which they are expressed (Wolff et al., 2004).

In recent years, studies have identified the localization of muscarinic acetylcholine (mAChRs) receptors, which are GPCRs composed of five subtypes, M_1-M_5. The M_1, M_3, and M_5 receptors couple to activation of phospholipase C via the $G_{q/11}$ family of proteins, whereas M_2 and M_4 receptors inhibit adenylyl cyclase via the $G_{i/o}$ family (Wess, 1996). The mAChr subtypes also have distinct subcellular distributions and are asymmetrically distributed in polarized cells such as pancreatic and lacrimal acinar cells, lingual epithelial cells, neuronal cells, and MDCK epithelial cells (Simon and Baggett, 1992; Toescu et al., 1992; Rouse and Levey, 1996; Nadler et al., 1999). Receptor chimera studies of mAChRs in MDCK cells have reported that M_3 receptors are targeted to the basolateral surface, while M_2 receptors are targeted to the apical surface.

Other examples of differentially localized GPCRs are the A1 and A2 adenosine receptors and the alpha$_2$-adrenergic receptors (α_2-AR). Utilizing surface biotinylation strategies and confocal microscopy, Saunders et al. (1995) determined that the adenosine A1 receptor is apically enriched in MDCK cells. Casavola and colleagues (1996) used frog kidney epithelial cells (A6) to show the A1 receptors are located on the apical surface, where they regulate apical Cl^- secretion via intracellular calcium, while A2 receptors are located on the basolateral surface, where they stimulate Na^+ transport via an increase in cAMP levels. Many studies have looked at the subcellular distribution patterns of the α_2-AR subtypes, α_{2A}, α_{2B}, and α_{2C}. The α_{2A}-AR and α_{2C}-AR are targeted to the basolateral surface, whereas the α_{2B}-AR is distributed randomly to both apical and

basolateral surfaces and then selectively retained at the basolateral membrane (Tan et al., 2004).

Evidence of Apical and Basolateral Sorting Signals in GPCRs

Additional studies have been done to determine the structural components of G protein-coupled receptors responsible for sorting these receptors to the apical or basolateral surface of polarized epithelial cells. While numerous studies suggest that GPCRs contain sorting signals within their sequence **(Table 2)**, the mechanisms responsible for GPCR sorting in polarized cells are relatively unknown. The current literature suggests that the sorting signals for a receptor are unique to the specific receptor. For example, receptors targeted to the apical surface of polarized epithelial cells may each contain a different sorting signal or structural motif responsible for targeting the receptor to the apical surface. Mutational studies have also determined specific amino acids or segments of the receptor sequence that are required for maintaining the localization of various GPCRs **(Table 3)**.

Table 2. Evidence of Apical and Basolateral Sorting Signals

Receptor	Apical vs Basolateral	Sorting Signal
$P2Y_2$	Apical	1st Extracellular loop
$P2Y_4$	Apical	C-terminal tail
M_2 Muscarinic	Apical	3rd Intracellular loop
M_3 Muscarinic	Basolateral	3rd Intracellular loop
$Alpha_{2A}$ Adrenergic	Basolateral	TM 1-5, TM 6-7
$Alpha_{2B}$ Adrenergic	Random	?

Table 3. Sorting Signals and Amino Acid Motifs

Sequence	Receptor	7TM Location	Localization
PLLVYYYA**RGD**HWPFSTVLC K**L**VRFLFYT *	$P2Y_2$	1^{st} EC Loop	Apical
^{250}VTENCVQGEEK^{280} ^{280}KESSNDSTSVSAVASNMR DDEITQDENTVSTSLGHSKDE NSKQTCIKIVTKTQKSDSCTP ANTTVELVGSS^{350}	M_2	3^{rd} IC Loop	Apical
SGTEA**E**TEN**FV**HPTGSSRSCS	M_3	3^{rd} IC Loop	Basolateral
DRHRAICRPMLAYRHGSGAW NRP	V2	2^{nd} IC Loop	Basolateral
RQGLHNMEDVYELIENSH	TSH	C-tail	Basolateral
VTSGST**Y**I**L**VPLSH	FSH	C-tail	Basolateral
RSTLKLTTLQCQYSTVMD	LH	C-tail	Basolateral

*Amino acids highlighted in red have been shown to play a critical role in receptor sorting.

Using M_2/M_3 muscarinic receptor chimeras, Nadler et al. (2000) discovered a 21-residue sequence in the third intracellular loop, Ser^{271}-Ser^{291}, which was determined to be the basolateral sorting signal in the M_3 receptor. This sequence, when added to the third intracellular loop or C-tail of the M_2 receptor, changed its localization from the apical surface to the basolateral surface. This also showed that the M_3 basolateral sorting signal is dominant over the apical sorting signal(s) located within the M_2 receptor. Later studies by Iverson et al. (2005) revealed the basolateral sorting signal is a seven-residue sequence located in the third intracellular loop. Chmelar and Nathanson (2006) recently demonstrated that there were two independent sequences within the third intracellular

loop of the M_2 receptor, Val^{270}-Lys^{280} and Lys^{280}-Ser^{350}, that controlled the apical sorting of that receptor. This study also demonstrated that the M_2 receptor was first sorted to the basolateral surface and before reaching the apical surface. This study was the first to show that a GPCR achieves apical localization in MDCK cells via transcytosis, which typically does not occur in this cell line.

Sorting signals have also been identified in the alpha adrenergic receptors. Truncations of the α_{2A}-AR and chimeras with the apically targeted A1 adenosine receptor revealed that α_{2A}-AR targeting to the basolateral surface relies on multiple membrane-embedded sequences within or near the lipid bilayer, suggesting that a three-dimensional surface provides the basis for interaction with trafficking molecules (Saunders et al., 1998). Additionally, α_{2A}-AR trafficking occurs independently of posttranslational receptor modification (Tan et al., 2004).

It is also important to note that while all three subtypes of the alpha adrenergic receptor are located on the basolateral surface of MDCK cells, they achieve this localization via different trafficking mechanisms. While the α_{2C}-AR shares the same basolateral localization characteristics as the α_{2A}-AR, it is not exclusively located on the cell surface during steady-state (Wozniak and Limbird, 1995). In contrast, the α_{2B}-AR, while exclusively located on the cell surface during steady-state, is first randomly distributed to both the apical and basolateral surfaces (Wozniak and Limbird, 1995).

In general, the localization of receptor subtypes to one of these two surfaces allows these cells to regulate a broad range of homeostatic functions, including the movement of water, ions, and nutrients between the lumen and underlying tissue. Sorting to a particular surface can occur by a variety of mechanisms involving receptor

intracellular domains or other sequences. Moreover, these models provide an opportunity to explore the mechanisms underlying prostaglandin E_2 receptor localization.

History of Prostaglandins

The physiological effects of prostaglandins were first reported in 1930 when two gynecologists from Columbia University, Kurzrok and Lieb, observed that human semen caused rhythmic contraction and relaxation of uterine smooth muscle. A few years later, British pharmacologist M.W. Goldblatt described the hypotensive actions of human semen (1933). At the same time, Swedish scientist U.S. von Euler discovered the vasodepressor activity of human seminal fluid (1934, 1935). This effect was also observed in prostate tissue extracts from animals, such as dogs and rabbits (Euler & Hammerström, 1937). Von Euler later showed that this substance could also cause smooth muscle contraction (1937). Through numerous studies, von Euler discovered a novel lipid soluble material within the seminal fluid he termed "prostaglandins" because of their presence in prostate extract.

Twenty years later in 1957, Sune K. Bergström of Sweden was the first to isolate prostaglandins from sheep vesicular glands using countercurrent fractionation and reversed-phase chromatography (Flower, 2006). The structures of two factors, termed PG E and PG F, were identified using the world's first gas chromatograph-mass spectrometer (Bergström and Sjövall, 1960a, b). By 1962, Bergström and his colleagues had isolated and identified the structures of six prostaglandins, which included prostaglandin E_1, E_2, E_3, $F_{1\alpha}$, $F_{2\alpha}$, and $F_{3\alpha}$. It was determined that all of these

prostaglandins consisted of twenty-carbon unsaturated carboxylic acids with cyclopentane rings, formed from unsaturated fatty acids, primarily arachidonic acid. Over the next two decades, Bergström, along with colleagues Bengt I. Samuelsson and John R. Vane discovered other local hormones closely related to prostaglandins, which included leukotrienes, prostacyclin and thromboxane.

Biosynthesis of Prostaglandins

Eicosanoids are signaling molecules derived from three different twenty-carbon essential fatty acids (EFAs) which contain 3, 4, or 5 double bonds: dihomo-gama-linolenic acid, arachidonic acid, and eicosapentaenoic acid, respectively. The eicosanoids are comprised of the prostaglandins, prostacyclins, thromboxanes and leukotrienes. Prostaglandins are primarily synthesized from arachidonic acid. Arachidonic acid is the most abundant precursor of eicosanoids in humans; however, it cannot be synthesized de novo. It is derived from the diet through linoleic acid or ingested as a dietary supplement.

In order for biosynthesis of eicosanoids to commence, activation of plasma membrane-bound receptors that couple to GTP-binding regulatory proteins (G proteins) must occur. Activation of these G protein-coupled receptors results in the activation of phospholipases (A_2 or C), which in turn mobilize arachidonic acid from membrane phospholipids by hydrolyzing the *sn*-2 ester bond. Several different forms of phospholipase A_2 mediate arachidonic acid release, including cardiac PLA_2, cytosolic PLA_2 (Lin et al., 1992), and secretory PLA_2 (Reddy et al., 1997). Arachidonic acid is

also released by the combined action of phospholipase C and diglyceride lipase (Katzung, 2004).

After mobilization, arachidonic acid is oxygenated by four separate routes: the cyclooxygenase (COX), lipoxygenase, P450 epoxygenase, and isoprostane pathways. The cyclooxygenase pathway produces prostacyclin, thromboxane and prostaglandins D, E and F. These products are collectively known as prostanoids. There are two unique COX enzymes responsible for converting arachidonic acid to prostanoids. Cyclooxygenase-1 (COX-1 or PGH synthase-1) is constitutively active, whereas cyclooxygenase-2 (COX-2 or PGH synthase-2) is inducible, or dependent upon a stimulus. COX-1 and COX-2 utilize endoperoxide synthase activity to oxygenate and cyclize arachidonic acid to yield the cyclic endoperoxide PGG_2. The enzymes then utilize peroxidase activity to convert PGG_2 to PGH_2. PGG_2 and PGH_2 are both chemically unstable; however, PGH_2 can be enzymatically converted to the prostanoids, including PGE, PGF, PGD, PGI and TXA, via separate pathways.

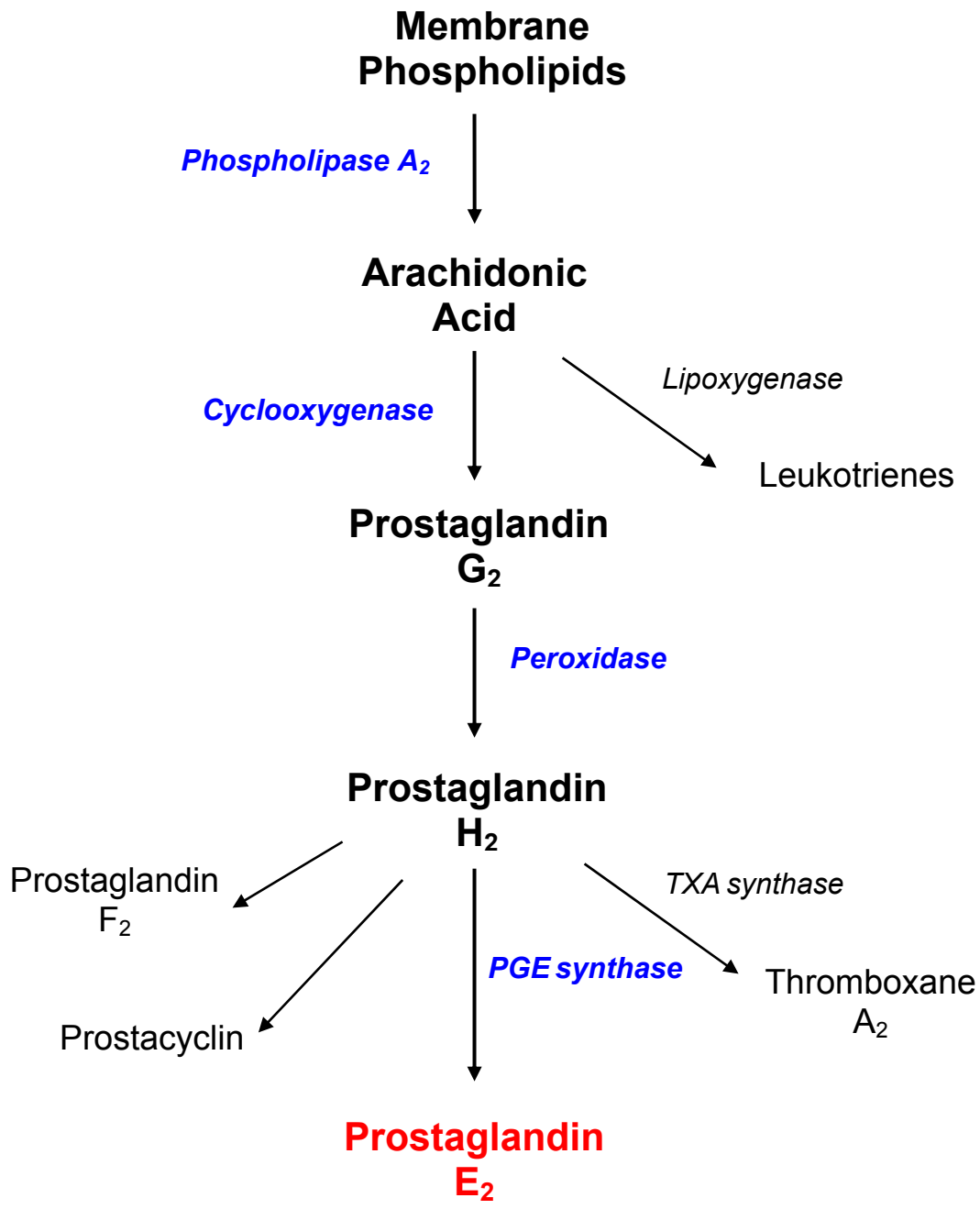


Figure 2. Synthesis of Eicosinoids from Arachidonic Acid Metabolism

Prostaglandins are synthesized from arachidonic acid in the cell membrane by the action of phospholipase A_2. The pathway splits and forms either prostaglandins and thromboxane or leukotrienes. The cyclooxygenase pathway yields PGE_2 from PGH_2 and the enzyme PGE synthase (from Katzung, 2004).

Pharmacological Actions and Clinical Significance of PGE_2

The human prostaglandin E_2 receptors are involved in numerous physiological functions as they are widely distributed throughout the body. PGE_2 has major effects on airway, gastrointestinal, reproductive and vascular smooth muscle. Interestingly, PGE_2 causes constriction of smooth muscle through mobilization of calcium or relaxation of smooth muscle through an increase in cAMP. The effects of PGE_2 also extend to the kidney, central and peripheral nervous systems, bone metabolism and eye.

In the kidney, PGE_2 is synthesized at high rates along the nephron (Bonvalet et al., 1987) where it is involved in the maintenance of normal renal blood flow and function during periods of physiological stress (Yared et al., 1985). Similar to its opposing effects in smooth muscle, PGE_2 can inhibit or stimulate epithelial salt and water transport in the nephron (Anderson et al., 1976). Many studies suggest that there are three distinct effects of basolateral PGE_2 on transport in the kidney: stimulation of basal water absorption; inhibition of vasopressin-stimulated water absorption; and inhibition of Na^+ absorption (Breyer and Breyer, 2000). The dual, opposing effects of PGE_2 on the maintenance of vascular tone, water absorption and sodium absorption signify that there are distinct EP receptors involved in these processes (Coleman et al., 1994; Toh et al. 1995).

In the female reproductive system, PGE_2 has potent oxytocic effects as it is able to terminate pregnancy at any stage by causing uterine contractions. This effect has been manipulated for clinical use through the PGE_2 analogue dinoprostone, which is used to induce labor by ripening the cervix and stimulating contractions or as an abortifacient. The effects of oral PGE_2 are similar to those of intravenous oxytocin; however, the only

formulation of dinoprostone available in the United States is for vaginal administration, which is less effective then oxytocin. In fact, oral PGE_2 would be preferred for women with preeclampsia or cardiac or renal complications over oxytocin due to its diuretic and natriuretic properties.

In the cardiovascular system, PGE_2, in addition to PGI_2, plays a major role in maintaining the patency of the ductus arteriosus. In neonates, the ductus arteriosus is a normal structure that allows most of the blood leaving the right ventricle to bypass pulmonary circulation and pass into the descending aorta. Following birth, this structure closes and allows for normal blood circulation through the lungs. Failure of this structure to close is termed patent ductus arteriosus. Clinically, cyclooxygenase inhibitors are often given to prevent prostaglandin synthesis and subsequently close the ductus.

Elsewhere in the body, PGE_2 also plays various roles. For example, in the gastrointestinal tract, PGE_2 protects the integrity of the gastric mucosa by defending against gastric irritants (Hoshino et al., 2003). PGE_2 potently modulates immune responses by inhibiting T cell proliferation, differentiation, membrane receptor expression, and the secretion of diverse cytokines (Geotzl et al., 1995). Other actions of PGE_2 range from temperature regulation to preservation of intraocular pressure (Mishima and Masuda, 1979; Ushikubi et al., 1998). Specific physiological effects of prostaglandin E_2 throughout the body are summarized in **Table 4**.

Table 4. Physiological Actions of PGE_2

Cell, Tissue or	Response	Reference
Endocrine System	↓ Insulin Secretion	(Cheng et al., 1993)
	↓/↑ Hypothalamic hormone secretion	(Ojeda et al., 2000)
Gastrointestinal System	↓ Acid secretion	(Newman et al., 1975)
	↑ Mucous secretion	(Bolton et al., 1976)
Immune System	↓ T and B cell function	(Wu et al., 2004)
	↓ Mast cell histamine release	(Guek et al., 2003)
Metabolic System	↓ Lipolysis	(Kather and Zimmer, 1983)
	↑ Bone formation	(Weinreb et al., 1999; Sakuma et al., 2000)
Nervous System	↑ Body temperature	(Bernheim et al., 1979)
	↑ Sensory afferent stimulation	(Herman et al., 1978)
	↓ Neurotransmitter release	(Malik and McGiff, 1975)
Platelets	Potentiates or inhibits aggregation	(Anderson et al., 1980; Shio and Ramwell, 1972)
Reproductive System	↑ Uterine contractions	(Katzung et al., 2004)
Renal System	↓ H_2O reabsorption	(Dunn and Hood, 1977)
	↓ Electrolyte reabsorption	(Stokes, 1979)
	↑ Renin release	(Keeton and Campbell, 1980)
Smooth Muscle (Vascular)		
Jugular Vein	Relaxation	(Lawrence et al., 1992)
Saphenous Vein	Relaxation	(Coleman et al., 1994)
Smooth Muscle (Nonvascular)		
Gastrointestinal	Contraction/Relaxation	(Coleman et al., 1987; Lawrence et al., 1992)
Trachea	Contraction/Relaxation	(Mathe et al., 1977)
Uterus	Relaxation	(Bergstrom et al., 1968)

Membrane Domain-Specific Effects of PGE_2

As previously mentioned, many tissues express several subtypes and isoforms of the EP receptor, suggesting a complex interplay among the receptors. Studies have shown that PGE_2 has membrane domain-specific effects. In the kidney, for example, where PGE_2 modulates salt and water transport in the distal tubule and stimulates renin release, studies preformed in isolated rabbit cortical collecting duct (CCD) indicate that PGE_2 has distinct effects on the luminal and basolateral surfaces (Hebert et al., 1993; Ando and Asano, 1995; Sakairi et al., 1995). Sakairi et al. (1995) showed luminal PGE_2 transiently hyperpolarized transepithelial voltage (Vt) in a dose-dependent manner, in contrast to a sustained depolarization of Vt produced by basolateral PGE_2. Sulprostone, a PGE_2 analogue selective for EP1 and EP3 receptors, affected Vt only when applied from the basolateral surface and not the luminal surface. Luminal application of the EP2 receptor agonist butaprost was also without effect. The results suggest that luminal PGE_2 affects Vt via a butaprost-insensitive EP4 receptor. The Vt effect of luminal PGE_2 was not blocked by pertussis toxin, also arguing against an EP3-mediated G_i-coupled effect. Luminal PGE_2 also suppressed sodium transport and stimulated osmotic water permeability (Ando and Asano, 1995; Sakairi et al., 1995). Sakairi et al. (1995) also observed rabbit cortical collecting ducts cultured on semipermeable supports. Interestingly, they observed apical PGE_2 stimulated cyclic AMP formation, suggesting that the effects of luminal PGE_2 are mediated by adenylyl cyclase-stimulating EP2 or EP4 receptors.

Receptors for PGE_2

The effects of prostaglandin E_2 are mediated by binding to cell surface G protein-coupled receptors. PGE_2 binds with similar affinity to four distinct receptor subtypes, designated EP1, EP2, EP3 and EP4. In addition, the EP3 receptor exists as 8 different C-tail isoforms. All of the receptors have been cloned and are distinguished by their second messenger signaling pathways **(Table 5)**. Tissue mRNA expression for these receptors also varies throughout the body, which may explain the numerous physiological functions of PGE_2.

Table 5. Signaling Pathways of PGE_2 Receptor Subtypes

EP Subtype	Signaling Pathways
EP1	**G_q** IP3/DAG/PKC
EP2	**G_s** ↑cAMP
EP3 (8 isoforms)	**G_i**, G_s, G_q, $G_{12/13}$ ↓cAMP
EP4	**G_s** ↑cAMP

*Abbreviations: IP3, inositol 1,4,5-triphosphate; DAG, diacylglycerol; PKC, Protein Kinase C.

While there are numerous physiological pathways activated by PGE_2, the receptors responsible for all of these effects have yet to be elucidated. There is a significant amount of literature on this topic and as a result, a summary including a few key functions for each PGE_2 receptor subtype is provided below.

EP1

The human EP1 receptor is a 402 amino acid protein, originally described as a smooth muscle constrictor (Funk et al., 1993). Functionally, EP1 preferentially couples to the activation of phospholipase C, causing an increase in intracellular calcium. These actions are mediated via the G_q pathway. A study by Narumiya et al. (1999) showed that upon agonist stimulation of the cloned mouse EP1 receptor, a strong increase in intracellular calcium concentration was observed. However, only a small increase in IP3 generation was observed, suggesting that the EP1 receptor may also function through an alternative mechanism independent from the G_q G protein. Evidence suggests that there is a C-terminal splice variant of the EP1 receptor present in the kidney and uterus (Okuda-Ashitaka et al., 1996). The mRNA expression levels for the EP1 receptor are low compared to the other subtypes. EP1 mRNA is located in the kidney, lung and stomach (Watabe et al., 1993).

Pharmacologically, there are no reported highly selective agonists for the EP1 receptor. Sulprostone was originally described as a potent EP1 agonist but was later determined to be a more potent EP3 agonist (Bunce et al., 1990). However, the selective agonists 17-phenyl-ω-trinor PGE_2 and iloprost have effectively been used to demonstrate the EP1-mediated effects of PGE_2 (Sheldrick et al., 1988: Lawrence et al., 1992). Numerous proprietary compounds have been shown to be selective antagonists for the EP1 receptor, including ONO-8711, SC 19220 and AH6809 (Woodward et al., 1995: Dey et al., 2006).

Studies using EP1 knockout mice suggest the EP1 receptor plays a direct role in mediating algesia and regulating blood pressure. Stock et al. (2001) showed these

animals display a 50 % reduction in pain-sensitivity responses. Additionally, a decrease in systolic blood pressure accompanied by increased renin-angiotensin activity was observed (Stock et al., 2001). The EP1 receptor has also been shown to mediate neurotransmitter release (Coleman et al., 1990). A recent study found that in the striatum PGE_2 is synthesized in response to dopamine receptor stimulation and, through the EP1 receptor, PGE_2 amplified both dopamine D1 and D2 receptor signaling (Kitaoka et al., 2007).

EP2

The human EP2 receptor is a 358 amino acid protein that couples to the G_s pathway, resulting in stimulation of adenylyl cyclase and increased cAMP levels (Bastien et al., 1994; Regan et al., 1994). Interestingly, Stillman et al. (1999) determined that the extracellular domains of this receptor play a critical role in its function. The EP2 receptor is the least abundant of the EP receptor subtypes; however, it is widely distributed in smooth muscle (Coleman et al., 1994). Northern blot analysis in mouse tissue has shown the EP2 receptor is highly expressed in the lung, spleen, heart and uterus (Honda et al., 1993). With regard to molecular regulation, the EP2 receptor is resistant to agonist-induced desensitization and internalization (Nishigaki et al., 1996; Desai et al., 2000). Pharmacologically, the EP2 receptor is distinguished from the other subtypes by the selective agonist butaprost (Gardiner et al., 1986). There are currently no selective antagonists for this receptor commercially available.

Studies suggest that the EP2 receptor is involved in blood pressure control and fertility. Kennedy et al. (1999) showed mice lacking the EP2 receptor displayed salt-

sensitive hypertension and that PGE_2 levels in the urine were increased in response to a high salt diet. They also reported reduced litter sizes due to impaired ovulation. Recent studies using EP2 knockout mice showed these receptors are involved with the protumorigenic action of PGE_2, as these mice displayed a decrease in skin tumor development (Sung et al., 2005).

EP3

The human EP3 receptor was originally described as a constrictor of smooth muscle (Coleman et al., 1994). In humans, at least eight EP3 receptor splice variants, forming eight isoforms, have been identified and several splice variants also exist in other species such as mouse, rabbit, and cow (Breyer et al., 1993; Irie et al., 1993; Namba et al., 1993). The human EP3 isoforms share an identical sequence over the first 359 amino acids but differ in the compositions of their C-tails, ranging in length from 6 to 66 amino acids **(Table 6)** (Kotani et al., 1997). All EP3 isoforms couple to the G_i pathway, resulting in the inhibition of adenylyl cyclase and decreased levels of cAMP. However, studies have also shown that the isoforms couple to G_i, G_q, and $G_{12/13}$ (An et al., 1994). It has been shown that the G protein specificity is determined by the cytoplasmic tail (Namba et al., 1993; Kotani et al., 1995).

Table 6. Comparison of Human EP3 Receptor Isoform C-tail Sequences

Receptor	C-tail Sequence
EP3.III	**FCQ**-EEFWGN*
EP3.IV	**FCQ**-MRKRRLREQEEFWGN*
EP.II	**FCQ**-VANAVSSCSNDGQKGQPISLSNEIIQTEA*
EP3.I	**FCQ**-IRYHTNNYASSSTSLPCQCSSTLMWSDHLER*
EP3.e	**FCQ**-MRKRRLREQLICSLRTLRYRGQLHIVGKYKPIVC*
EP3.VI	**FCQ**-EMGPDGRCFCHAWRQVPRTWCSSHD**REPCSVQLS***
EP3.V	**FCQ**-MRKRRLREQEMGPDGRCFCHAWRQVPRTWCSSHD REPCSVQLS*
EP3.f	**FCQ**-MRKRRLREQAPLLPTPTVIDPSRFCAQPFRWFLDLSFP AMSSSHPQLPLTLASFKLL**REPCSVQLS***

The EP3 receptor is widely distributed throughout the body, found in smooth muscle of the gastrointestinal tract, uterus, and vascular tissues. Additionally, the EP3 receptor is highly expressed in the kidney and gastric mucosa (Breyer et al., 1993; Sugimoto et al., 1994). Findings by Kotani et al. (1995) indicate the isoform mRNAs are expressed in a tissue-specific manner, suggesting that there are multiple physiological effects of PGE_2 that are mediated by EP3 isoforms. EP3 receptors are known to play a role in modulating fever. Knockout mice lacking the EP3 receptor failed to show a febrile response to PGE_2 and IL-1β, exogenous and endogenous pyrogens respectively (Ushikubi et al., 1998). EP3 knockout mice have also been used to demonstrate the role of EP3 receptors in the stomach. Nishio and colleagues (2007) showed the inhibitory action of PGE_2 on gastric acid secretion in a damaged stomach was mediated by the EP3 receptor.

A number of functional differences between the isoforms have been reported. These include differential activation of signal transduction pathways (Irie et al., 1993; Namba et al, 1993; Sugimoto et al., 1993; Kotani et al., 2000) and agonist-independent constitutive activity (Hasegawa et al., 1996; Jin et al., 1997). Previous studies from our laboratory determined that the EP3 isoforms have tissue-specific distribution patterns, as well as different agonist-induced internalization patterns in HEK293 cells using confocal microscopy (Bilson et al., 2004). We have shown that some isoforms internalize completely, whereas other isoforms internalize to a lesser degree or not at all. Isoform I internalized completely, isoforms II, V, VI and f internalized to a lesser extend, and isoforms III and IV did not internalize at all **(Figure 3)**.

Evidence suggests that the isoforms also vary in their subcellular expression. Studies in cultured MDCK cells show that the mouse EP3 isoforms have distinct subcellular localization patterns. Hasegawa and coworkers (2000) used MDCK cells, which are generally used as a model of polarized epithelial cells, to show that the mouse EP3γ receptor is preferentially targeted to the lateral plasma membrane. The EP3γ receptor is homologous to the human EP3.II receptor isoform. It is also interesting to note that the composition of the C-tail of EP3γ appears to determine its targeting (Hasegawa et al., 2000).

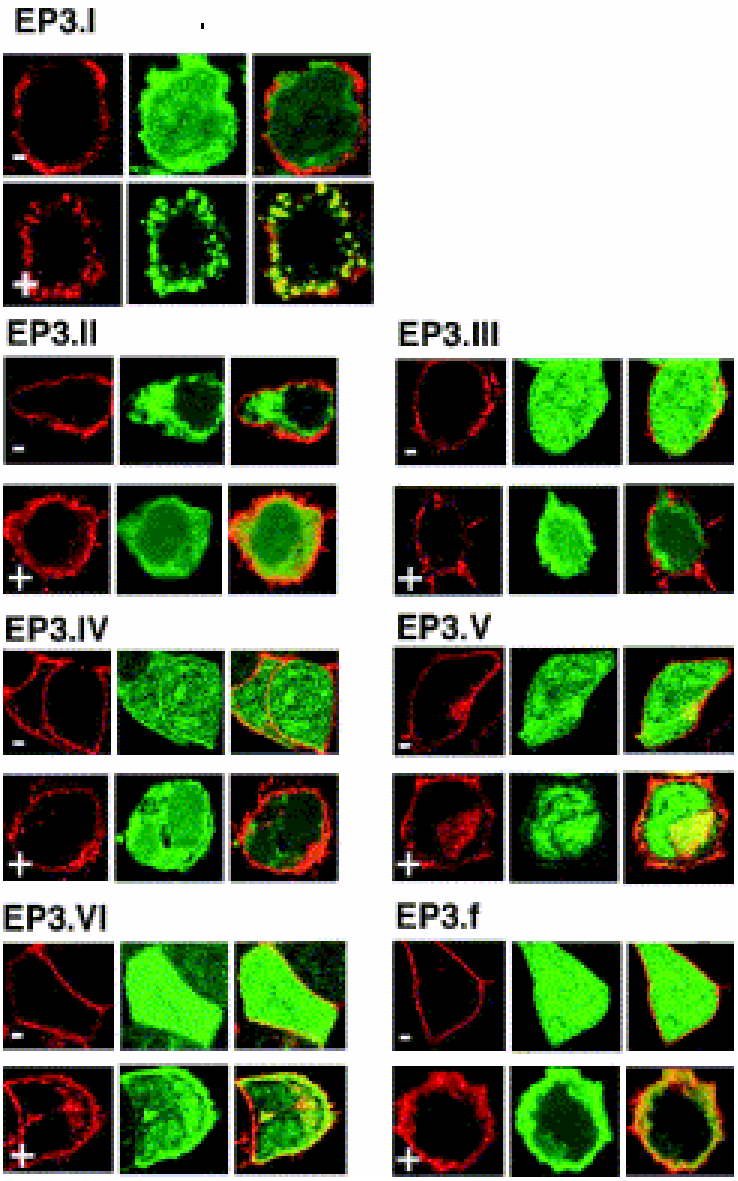


Figure 3. Agonist-Induced Internalization Patterns of the Human Prostaglandin EP3 Isoforms (Bilson et at., 2004)

EP4

Similar to the EP2 receptor, the human EP4 receptor, a 488 amino acid protein, signals through the G_s pathway to stimulate adenylyl cyclase and increase cAMP (Bastien et al., 1994; Regan et al., 1994). While these two receptors mediate their effects through the same pathway, several differences exist between them. Although both receptors increase levels of cAMP, it has been shown that the EP4 receptor demonstrates much weaker coupling to G_s compared to the EP2 receptor (Honda et al., 1993; Regan et al., 1994; Regan, 2003). Some studies indicate that G_s-mediated increases in cAMP levels play a less important role in EP4 receptor signaling compared with EP2 receptor signaling. For example, the EP4 receptor mediates proliferation of colon carcinoma cells in the absence of detectable increases in cAMP (Sheng et al., 2001; Mutoh et al., 2002). Functional differences in EP2 and EP4 receptor signaling may also arise from different agonist-induced desensitization and internalization patterns. Compared to the EP2 receptor, the EP4 receptor has a much longer C-tail region, which is required for rapid agonist-induced desensitization (Nishigaki et al., 1996; Bastepe and Ashby, 1997). In addition, the difference in C-tail length has been shown to be responsible for the observation that the EP4 but not EP2 receptor undergoes agonist-induced internalization (Desai et al., 2000; Desai and Ashby, 2001).

The EP4 receptor is widely distributed throughout the body. EP4 receptor mRNA is expressed in kidney, thymus, ileum, adrenal gland, lung and spleen (Bastien et al., 1994). Pharmacologically, it is distinguished from the EP2 receptor by its insensitivity to butaprost (Nishigaki et al., 1995).

Studies have shown that EP4 is involved in numerous physiological functions. The EP4 receptor is known to play a critical role in maintaining the patency of the ductus arteriosus. EP4 knockout mice were used to demonstrate that proper function of this receptor is essential for neonatal adaptation of the circulatory system (Segi et al., 1998). Mice engineered to be lacking the EP4 receptor in cardiac myocytes showed the EP4 receptor played a role in hypertrophy (Qian et al., 2008). The EP4 receptor has also been shown to function in endothelial cell migration (Rao et al., 2007) and maintaining bone mass and fracture healing (Li et al., 2005). Additional studies have shown involvement of the EP4 receptor in mammary tumor metastasis *in vivo* (Ma et al., 2006).

Purpose of Thesis

The purpose of this thesis is to examine the subcellular localization patterns of the human prostaglandin E_2 receptors in polarized epithelial cells. These cells contain distinct apical and basolateral plasma membrane domains with specific protein compositions, separated by tight junctions (Rodriguez-Boulan et al., 1989). Protein sorting to specific membranes in epithelial cells is critical for proper cell function; however, the mechanisms behind this are poorly understood. We hypothesized that the human EP receptors would display unique patterns of localization in polarized epithelial cells and that these differences may correspond to the various physiological functions of these receptors.

We have examined the localization patterns of the human EP receptors in two different epithelial cell lines using retroviral expression. The cell lines used were Madin-

Darby canine kidney (MDCK) and human bronchial epithelial (BEAS-2B) cells. We have determined unique localization patterns for EP2, EP4, EP3.I, EP3.II, EP3.VI and EP3.f. Our results indicate that there is no significant difference in localization of these receptors in kidney and lung cells. Additionally, we have used one mutated form of the EP3.I receptor and two mutant forms of the EP4 receptor to determine the significance of serine and threonine residues within the C-tails in relation to the sorting of these receptors. The localization patterns for each receptor, isoform and mutant were obtained using confocal immunofluorescence microscopy.

Studies using receptor chimeras determined structural components responsible for apical or basolateral sorting. We utilized two apically sorted purinergic receptors, $P2Y_2$ and $P2Y_4$, which contain known sorting signals within the first extracellular loop and C-terminal tail, respectively. Chimeras were constructed using the basolaterally localized EP3.VI receptor. These constructs were created to contain the truncated purinergic receptor and the EP3.VI C-tail. The reverse constructs of the truncated EP3.VI receptor and purinergic $P2Y_2$ or $P2Y_4$ C-tail were also generated.

Overall, the aim of this thesis is to further examine the differences among the human prostaglandin E_2 receptors by observing their localization patterns in polarized epithelial cells. We have shown unique patterns for various receptors and observed consistency in two different cell lines. While we have confirmed that the C-tail of the EP3 isoforms play a role in receptor localization, the localization patterns of our chimeras suggest that there may be additional structural components of the receptor that are involved in trafficking the receptors to specific membrane domains. Further studies in this area may determine the role of receptor localization in receptor function, as this may

lead to a more complete understanding of the physiological role of the various human prostaglandin E_2 receptors.

CHAPTER 2

EXPERIMENTAL PROCEDURES

Materials

PGE_2 Receptor DNA

cDNAs for EP2, EP3.I, EP3.II, EP3.VI, EP3.f, and EP4 were previously obtained by RT-PCR in this laboratory (Bastepe and Ashby, 1997; Desai et al., 2000; Bilson et al., 2004). They were epitope-tagged with an N-terminal hemagluttinin (HA) tag and inserted into the vector pcDNA3.1/Zeo (-) (Desai et al., 2000; Bilson et al., 2004).

Purinergic Receptor DNA

Purinergic $P2Y_2$ and $P2Y_4$ receptor constructs were purchased from the UMR cDNA Resource Center (Rolla, MO).

DNA Constructs and Purification

Custom primers and MAX Efficiency Stbl2 competent *E. coli* cells were purchased from Invitrogen (Carlsbad, CA). The *PfuTurbo* DNA Polymerase, SURE 2 Supercompetent *E. coli* cells, pBluescript II phagemid vector and pFB-neo retroviral vector were purchased from Stratagene (La Jolla, CA). QIAquick and MinElute DNA gel extraction/PCR purification kit and QIAfilter Plasmid Maxi Kit were purchased from Qiagen (Valencia, CA). The Wizard Plus Miniprep DNA Purification System was purchased from Promega (Madison, WI). The Rapid DNA Ligation kit was purchased from Roche (Mannheim, Germany).

Cells and Cell Culture

PA317 packaging cells, Madin-Darby canine kidney (MDCK) cells and human bronchial epithelial (BEAS-2B) cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). DMEM, DMEM/F-12, and RPMI 1640 medium and Geneticin (G418) were from Gibco BRL (an Invitrogen corporation) (Carlsbad, CA). Fetal Bovine Serum and Antibiotic-Antimycotic Solution were purchased from Cellgro (Manassas, VA). Transwell permeable supports were purchased from Corning (Lowell, MA). Tissue culture dishes, bacterial plates, and supplies were from Fischer Scientific (Pittsburgh, PA), while all other chemicals and reagents were purchased Invitrogen.

Antibodies

The anti-HA monoclonal antibody (HA.11) was purchased from Covance (Berkeley, CA) and the anti-zonula occludens (ZO) polyclonal antibody was purchased from ZYMED Laboratories (an Invitrogen corporation) (Carlsbad, CA). Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 goat anti-mouse antibodies were purchased from Molecular Probes (an Invitrogen corporation) (Eugene, OR).

Construction of Retroviral Vectors Containing PGE_2 Receptors

cDNAs for EP2, EP3.I, EP3.II, EP3.VI, EP3.f, and EP4 have previously been prepared by RT-PCR and are epitope-tagged with an N-terminal hemagluttinin (HA) tag. The receptors were first subcloned into the pBluescript II KS (+/-) vector. The PGE_2 receptors and pBluescript vector were digested with the restriction enzymes EcoRI and

HindIII with Multi-Core Universal Buffer for 1 hour at 37°C. The products were run on a 1.4% agarose gel and then purified using a DNA purification gel extraction kit. Each receptor insert was then ligated into the pBluescript vector using the Rapid DNA Ligation Kit. In summary, 7µL of insert and 1µL of vector were combined with 2µL of DNA dilution buffer. This mixture was then added to 10µL of DNA ligation buffer. Finally, 1µL of T4 DNA ligase was added and the mixture was incubated at room temperature for 10 minutes.

Each ligation was transformed into SURE2 Supercompetent *E. coli* cells from Stratagene. Resulting DNA was then extracted using a Wizard Plus Miniprep DNA Purification System. Plasmids were screened for inserts using restriction enzymes EcoRI and HindIII and their concentrations were determined using a spectrophotometer. Plasmids were then sequenced by SeqWright (a Fischer Scientific corporation) (Houston, TX).

Receptors were next cloned into the pFB-neo retroviral vector. Each receptor-pBluescript construct and the pFB-neo vector were digested with EcoRI and XhoI with Buffer D for 1 hour at 37°C. As described above, the products were run on a 1.4% agarose gel and then purified using a DNA purification gel extraction kit. Each receptor was then ligated in-frame into the pFB-neo vector using the Rapid DNA Ligation Kit. Each ligation was transformed into MAX Efficiency Stbl2 *E. coli* cells. Resulting DNA was then extracted from the bacterial cells using a Wizard Plus Miniprep DNA Purification System. Plasmids were screened for inserts using restriction enzymes EcoRI

and XhoI and their concentrations were determined using a spectrophotometer. Plasmids were then sequenced by SeqWright.

DNA for pFB-neo constructs was amplified by re-transformation into Stbl2 *E. coli* cells and then extracted using QIAfilter Plasmid Maxi Kit. Concentrations were then measured using a spectrophotometer.

Construction of Retroviral Vectors Containing PGE_2 Receptor Mutants

hEP3.I-5A Mutation

To determine the role of serine and threonine residues in receptor localization, mutations were made to the SSSTS sequence located in the EP3.I receptor C-tail. Using the QuikChange XL Site-Directed Mutagenesis Kit (Strategene), the five amino acid sequence was mutated to five alanines **(Figure 4)**. The resulting mutant DNA was ligated into the pcDNA3.1(+) vector.

Because there were no compatible restriction sites between the mutant construct and the pFB-neo retroviral vector, the hEP3.I-5A mutant receptor was first subcloned into the pBluescript II KS (+/-) vector. The hEP3.I-5A mutant receptor and pBluescript vector were digested with the restriction enzymes EcoRI and HindIII with Multi-Core Universal Buffer for 1 hour at 37°C. The products were run on a 1.4% agarose gel and then purified using the QIAquick Gel Extraction kit. The EP3.I-5A mutant receptor insert was then ligated in-frame into the pBluescript vector using the Rapid DNA Ligation Kit. In summary, 7µL of insert and 1µL of vector were combined with 2µL of DNA dilution buffer. This mixture was then added to 10µL of DNA ligation buffer.

Finally, 1μL of T4 DNA ligase was added and the mixture was incubated at room temperature for 10 minutes. This ligation was then transformed into MAX Efficiency Stbl2 *E. coli* cells. Resulting DNA was then extracted using a Wizard Plus Miniprep DNA Purification System. Plasmids were screened for inserts using restriction enzymes EcoRI and HindIII and their concentrations were determined using a spectrophotometer. Plasmids were then sequenced by SeqWright.

The hEP3.I-5A mutant receptor was next cloned into the pFB-neo retroviral vector. The hEP3.I-5A-pBluescript construct and the pFB-neo vector were digested with EcoRI and XhoI with Buffer D for 1 hour at 37°C. As described above, the products were run on a 1.4% agarose gel and then purified using a DNA purification gel extraction kit. The receptor was then ligated in-frame into the pFB-neo vector using the Rapid DNA Ligation Kit. The ligation was transformed into MAX Efficiency Stbl2 *E. coli* cells. The resulting DNA was then extracted from the bacterial cells using a Wizard Plus Miniprep DNA Purification System. Plasmids were screened for inserts using restriction enzymes EcoRI and XhoI and their concentrations were determined using a spectrophotometer. Plasmids were then sequenced by SeqWright.

The DNA for the hEP3.I-5A-pFB-neo construct was amplified by re-transformation into Stbl2 *E. coli* cells and then extracted using QIAfilter Plasmid Maxi Kit to increase DNA concentration. Concentrations were then measured using a spectrophotometer.

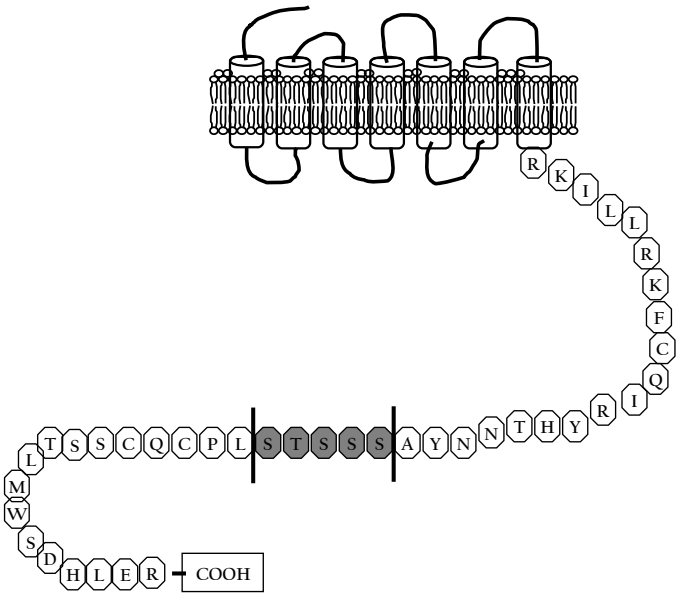


Figure 4. Schematic Representation of the EP3.I-5A Receptor Mutation

The SSSTS sequence located at amino acids 369 to 373 in the C-tail of the human EP3.I receptor was mutated to AAAAA as represented by the darkened circles.

hEP4-10ST and hEP4-t350 Mutations

The role of serines and threonines in receptor localization was also determined using two mutant forms of the human EP4 receptor **(Figure 5)**. For the hEP4-10ST mutation, 10 serines and threonines located within the C-tail sequence of the EP4 receptor were mutated to alanines. The hEP4-t350 mutant was truncated at amino acid 350 to remove 138 amino acids from the carboxyl terminal end of the receptor. The receptor mutants were previously prepared by Bastepe and Ashby (1999) using the overlapping PCR method according to Higuchi et al. (1988). Constructs were subsequently epitope-tagged with a hemagluttinin tag by Desai et al. (2000).

In our work, the forward primer for both mutations was AGG **GAA TTC** ATG TAC CCA TAC GAC GTC CCA GAC TAC GCT *TCC ACT CCC GGG GTC AAT*. The EcoRI restriction site is noted in bold while the HA tag sequence is in italics. The reverse primer for hEP4-t305 was GGA **GGA TCC** TAT TTA TTC AGC GGC AGA AGA GGC ATT TG. The reverse primer for EP4 10ST was GGA **GGA TCC** TTA TAT ACA TTT TTC TGA TAA GTT CAG TGT. Both reverse primers contained a BamHI restriction site noted in bold.

PCR amplification of each mutant was carried out using *PfuTurbo* DNA Polymerase. The products were purified and digested with EcoRI and BamHI with Multi-Core Universal Buffer for 2 hours at 37°C. The pFB-neo retroviral vector was also digested under the same conditions. The products were run on a 1.4% agarose gel and purified using the MinElute Gel Extraction kit. Each mutation was then ligated into the pFB-neo retroviral vector using the Rapid DNA Ligation kit. In brief, 7 μL of the

receptor insert and 1 μL of vector were combined with 2 μL of DNA dilution buffer. The mixture was added to 10 μL of T4 DNA ligation buffer and 1 μL of T4 DNA ligase. The reaction was allowed to incubate for 1 hour at room temperature.

Following the ligation, 1 μL of the reaction was transformed into MAX Efficiency Stbl2 *E. coli* cells. The resulting DNA was then extracted from the bacterial cells using a Wizard Plus Miniprep DNA Purification System. Plasmids were screened for inserts using restriction enzymes EcoRI and BamHI and their concentrations were determined using a spectrophotometer. Plasmids were then sequenced by SeqWright. In order to increase DNA concentrations, the constructs were further amplified by re-transformation into Stbl2 *E. coli* cells and then extracted using QIAfilter Plasmid Maxi Kit. Concentrations were then measured using a spectrophotometer.

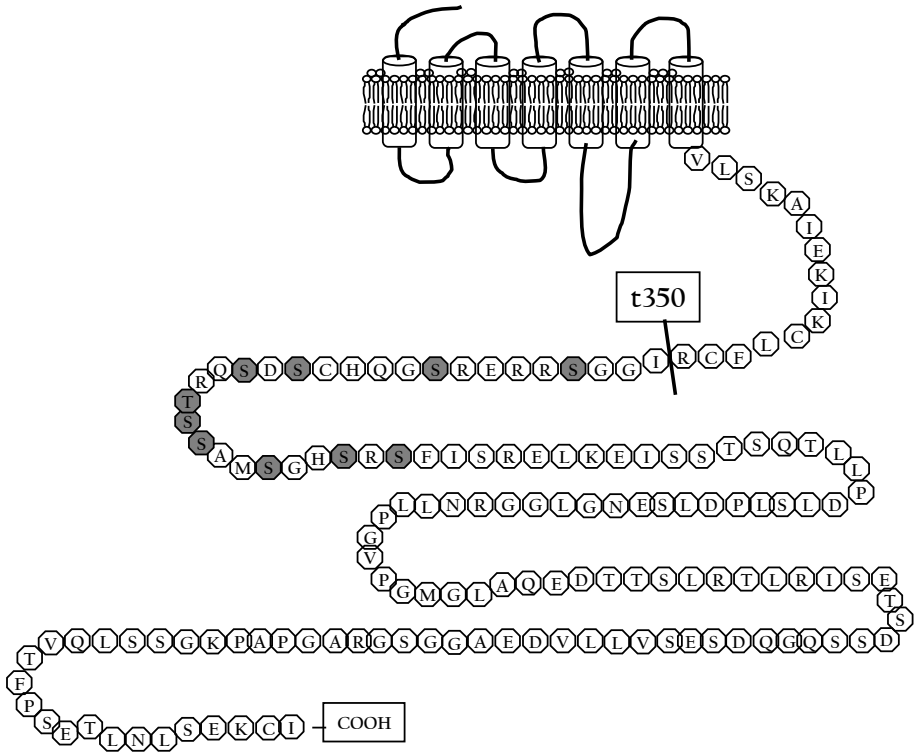


Figure 5. Schematic Representation of the EP4 Receptor Truncation and Mutation Positions

The human EP4 receptor was truncated at amino acid 350 to remove 138 amino acid residues from the carboxyl terminal end of the receptor. This truncation is referred to as the hEP4-t350 mutation. The darkened circles represent serine and threonine residues that were mutated to alanine for the hEP4-10ST mutation.

Preparation of Virus Particles Expressing the Receptor Constructs

Cell Culture

PA317 packaging cells were grown in Dulbecco's Modified Eagle Medium (DMEM) plus 10% fetal bovine serum and 2% Antimycotic Antibiotic. MDCK cells were grown in Dulbecco's Modified Eagle Medium (DMEM)/F-12 (1:1) plus 10% fetal bovine serum and 2% Antimycotic Antibiotic. BEAS-2B cells were grown in RPMI containing 10% fetal bovine serum and 2% Antimycotic Antibiotic. Cells were maintained in a humidified incubator at 37°C with 5% CO_2-95% air.

Calcium Phosphate Transfection

Recombinant retroviral particles were produced by calcium phosphate transfection of PA317 packaging cells with pFB-Neo vectors containing HA-tagged human PGE_2 receptor constructs. PA317 cells were grown in DMEM plus 10% FBA and 2% Antibiotic-Antimycotic Solution (complete medium) to 60% confluence in 60 mm dishes. Cells were transiently transfected with 20 μg of their respective DNA. Cells were incubated at 37°C for 24 hours. Twenty four hours post-transfection, cell medium was replaced with 3 mL complete medium plus 30 μL sodium butyrate and placed at 32°C for 48 hours. Recombinant retroviral particles were harvested and filtered using 0.45 μ filters and stored at -80°C.

Retroviral Transduction

MDCK or BEAS-2B cells were grown to 60% confluence in a 6-well plate. One milliliter of virus plus 0.8 μg/mL polybrene was then placed into its respective well. Cells were incubated with virus for 2 hours at 37°C. The virus particles were then removed and replaced with 2 mL of fresh complete medium. The cells were allowed to incubate in a humidified incubator at 37°C with 5% CO_2-95% air for 48 hours. Cells containing the geneticin-resistant retroviral particles were selected after 48 hours using complete medium containing 1 mg/ml G418.

Confocal Immunofluorescence Microscopy

MDCK cells stably expressing PGE_2 receptor retroviral constructs were cultured in DMEM/F-12 supplemented with 10% FBS, 2% Antibiotic-Antimycotic Solution, and 1 mg/mlG418 at 37ºC, 5% CO_2 on polycarbonate membrane filters (Transwell Permeable Supports, 0.4μ pore size). BEAS-2B cells stably expressing PGE2 receptor retroviral constructs were cultured in RPMI 1640 supplemented with 10% FBS, 2% Antibiotic-Antimycotic Solution, and 1 mg/mlG418 at 37ºC, 5% CO_2 on polycarbonate membrane filters (Transwell Permeable Supports, 0.4μ pore size) pretreated for 2 hours with collagen. Cells were maintained for 5-7 days on the transwell filters to allow for polarization.

Following polarization, cells were washed 3X with cold phosphate buffered saline (PBS), fixed and permeabilized with -20ºC methanol for 4 minutes, and then blocked with 5% non-fat dry milk in PBS for 30 minutes. Cells were treated with anti-HA

monoclonal antibody (1:500) and anti-zonula occludens (ZO) polyclonal antibody (1:100) in DMEM/F-12 or RPMI 1640 with 1% bovine serum albumin (BSA) for 60 minutes at room temperature. Cells were washed 3X with cold PBS and incubated with goat anti-mouse Alexa Fluor 594 and goat anti-rabbit Alex Fluor 488-conjugated antibodies (1:150) in 5% non-fat dry milk in PBS for 60 minutes at 37ºC. Cells were washed 3X with PBS and the transwell membranes were excised and mounted on glass slides with coverslips. Images were obtained using a Leica TCS-NT confocal microscope.

Construction of Receptor Chimeras

Full Length $P2Y_2$ and $P2Y_4$ Receptors

In order to create receptor chimeras, the full length purinergic $P2Y_2$ and $P2Y_4$ receptors in pcDNA3.1+ were first ligated into the pFB-neo retroviral vector. Each receptor was epitope-tagged with a hemagluttinin (HA) tag in order to be detected with a commercially available anti-HA monoclonal antibody. The Kozak consensus sequence (GCC GCC ACC) was also incorporated upstream of the HA tag in order to enhance expression levels. The sequence of the forward primer for $P2Y_2$ was GGA **GAA TTC** GCC GCC ACC ATG *TAC CCA TAC GAC GTC CCA GAC TAC GCT* GCA GCA GAC CTG GGC CCC TGG, with the EcoRI restriction site in bold and the HA tag indicated in italics. The reverse primer for $P2Y_2$ was GGA **CTC GAG** CTA CAG CCG AAT GTC CTT AGT GTT CTC GCT, with the XhoI restriction site in bold. The sequence of the forward primer for $P2Y_4$ was GGA **GAA TTC** GCC GCC ACC ATG TAC CCA *TAC*

GAC GTC CCA GAC TAC GCT GCC AGT ACA GAG TCC TCC CTG, with the EcoRI restriction site in bold and the HA tag indicated in italics. The reverse primer for P2Y4 was GGA **CTC GAG** TTA CAA TCT ATC TGC CCT AGG AGT AGA GCA, with the XhoI restriction site in bold.

Following amplification with PCR, the products were digested with EcoRI and XhoI using Buffer D for 1 hour at 37°C. The products were run on a 1.4% agarose gel, then purified using a MinElute Gel Extraction kit and ligated into the pFB-neo retroviral vector using the Rapid DNA Ligation kit. Each ligation was transformed into MAX Efficiency Stbl2 *E. coli* cells. Resulting DNA was then extracted from the bacterial cells using a Wizard Plus Miniprep DNA Purification System. Plasmids were screened for inserts using restriction enzymes EcoRI and XhoI and their concentrations were determined using a spectrophotometer. Plasmids were then sequenced by SeqWright.

$P2Y_2$-EP3.VI Chimeras

In order to create receptor chimeras between the purinergic $P2Y_2$ and the EP3.VI receptor, we had to incorporate a BamHI restriction site within the C-tail of the $P2Y_2$ receptor. The EP3.VI receptor has an internal BamHI site located at amino acid 341 in its C-tail. The truncated $P2Y_2$ receptor was amplified by PCR from cDNA using a forward primer containing the EcoRI restriction site and a reverse primer containing the BamHI restriction site. The forward primer was GGA **GAA TTC** GCC GCC ACC ATG TAC CCA *TAC GAC GTC CCA GAC TAC GCT* GCA GCA GAC CTG GGC CCC TGG. The forward primer contained the EcoRI restriction site noted in bold, an HA tag noted in

italics and also contained a Kozak sequence (GCC GCC ACC) in order to enhance expression. The reverse primer for the $P2Y_2$ truncated receptor was CAC G**GG ATC** **C**AG GCA ACT GTT and contained a BamHI restriction site noted in bold. PCR amplification was carried out using *PfuTurbo* DNA Polymerase. The truncated $P2Y_2$ PCR product was cut using EcoRI and BamHI restriction enzymes with Multi-Core Universal Buffer for 1 hour at 37°C. The EP3.VI receptor was also digested under the same conditions. The products were then run on a 1.4% agarose gel and the appropriate bands were purified using the MinElute Gel Extraction kit. A three-way ligation was then used to combine the truncated $P2Y_2$ receptor with the EP3.VI C-tail in the pcDNA3.1+ vector. To summarize, 3 μL of the truncated $P2Y_2$ receptor and 4 μL of the EP3.VI C-tail were combined with 1 μL of pcDNA3.1+ (digested with EcoRI and XhoI and gel purified) and 2 μL of DNA dilution buffer. This mixture was then added to 10 μL of T4 DNA ligation buffer and 1 μL of T4 DNA ligase. The reaction was then allowed to incubate at room temperature for 3 hours. After transformation of 1 μL of the ligation reaction into *E. coli* using Stbl2 cells, the resulting DNA was purified using the Wizard Plus Miniprep DNA Purification System. Insert-containing plasmids were identified using restriction analysis with EcoRI and XhoI with Buffer D.

Following gel purification, the $P2Y_2$-EP3.VI receptor insert was then ligated into the pFB-neo retroviral vector using the Rapid DNA Ligation kit. Next, 1 μL of the ligation reaction was transformed into Stbl2 *E. coli* cells and purified using the Wizard Plus Miniprep Purification System. Plasmids were screened for inserts using restriction enzymes EcoRI and XhoI and their concentrations were determined using a

spectrophotometer. Plasmids were then sequenced by SeqWright. DNA for pFB-neo constructs was amplified by re-transformation into Stbl2 *E. coli* cells and then extracted using QIAfilter Plasmid Maxi Kit. Concentrations were then measured using a spectrophotometer.

EP3.VI-P2Y$_2$ Chimera

The C-tail of the P2Y$_2$ receptor was amplified by PCR from cDNA using a forward primer containing the BamHI restriction site and a reverse primer containing the XhoI restriction site. The forward primer was AGT TGC CT**G GAT CC**C GTC CTC TAC TTC CTG with the BamHI restriction site in bold. The reverse primer sequence was GGA **CTC GAG** CTA CAG CCG AAT GTC CTT AGT GTT CTC GCT with the XhoI restriction site in bold. PCR amplification was carried out using *PfuTurbo* DNA Polymerase. The P2Y$_2$ C-tail PCR product was cut using BamHI and XhoI restriction enzymes with Buffer C for 1 hour at 37°C. The EP3.VI receptor was also digested under the same conditions. The products were then run on a 1.4% agarose gel and the appropriate bands were purified using the MinElute Gel Extraction kit.

A three-way ligation was then used to combine the truncated EP3.VI receptor with the P2Y$_2$ C-tail in the pFB-neo vector. To summarize, 6 µL of the P2Y$_2$ C-tail and 2 µL of the EP3.VI receptor in pFB-neo were combined with 2 µL of DNA dilution buffer. This mixture was then added to 10 µL of T4 DNA ligation buffer and 1 µL of T4 DNA ligase. The reaction was then allowed to incubate at room temperature for 3 hours. After transformation of 1 µL of the ligation reaction into *E. coli* using Stbl2 cells, the resulting

DNA was purified using the Wizard Plus Miniprep DNA Purification System. Insert-containing plasmids were identified using restriction analysis with EcoRI and XhoI with Buffer D and their concentrations were determined using a spectrophotometer. Plasmids were then sequenced by SeqWright. DNA for pFB-neo constructs was amplified by re-transformation into Stbl2 *E. coli* cells and then extracted using QIAfilter Plasmid Maxi Kit. Concentrations were then measured using a spectrophotometer.

Table 7. Size (bp) of EP3.VI and $P2Y_2$ Receptors, C-tails and Chimeras

Receptor	Size (base pairs)
EP3.VI Truncated Receptor	1023
EP3.VI C-tail	156
$P2Y_2$ Truncated Receptor	908
$P2Y_2$ C-tail	225
EP3.VI-$P2Y_2$ Chimera	1248
$P2Y_2$-EP3.VI Chimera	1064

$P2Y_4$-EP3.VI and EP3.VI-$P2Y_4$ Chimeras

In order to create receptor chimeras between the purinergic $P2Y_4$ and the EP3.VI receptor, we were able to utilize internal BamHI restriction sites within both receptors. The EP3.VI receptor has an internal BamHI site located at amino acid 1023 in its C-tail. The $P2Y_4$ receptor has an internal BamHI site located at amino acid 903 in its C-tail. Using the full-length receptor in the pFB-neo vector, the C-tails for each receptor were cut from the plasmid using the restriction enzymes BamHI and XhoI with Buffer C and digested overnight at 37°C. The digest was run on a 1.4% agarose gel and the high

molecular weight receptor-vector band and low molecular weight C-tail bands were cut and purified using the MinElute Gel Extraction kit. The $P2Y_4$ C-tail was ligated into the EP3.VI receptor and the EP3.VI C-tail was ligated into the $P2Y_4$ receptor. Using the Rapid DNA Ligation kit, 7μL of insert (C-tail) and 1μL of vector (containing the receptor) were combined with 2μL of DNA dilution buffer. This mixture was then added to 10μL of DNA ligation buffer. Finally, 1μL of T4 DNA ligase was added and the mixture was incubated at room temperature for 10 minutes. Each ligation was transformed into MAX Efficiency Stbl2 *E. coli* cells. Resulting DNA was then extracted from the bacterial cells using a Wizard Plus Miniprep DNA Purification System. Plasmids were screened for inserts using restriction enzymes BamHI and XhoI and their concentrations were determined using a spectrophotometer. Plasmids were then sequenced by SeqWright.

Table 8. Size (bp) of EP3.VI and $P2Y_4$ Receptors, C-tails and Chimeras

Receptor	Size (base pairs)
EP3.VI Truncated Receptor	1023
EP3.VI C-tail	156
$P2Y_4$ Truncated Receptor	906
$P2Y_4$ C-tail	192
EP3.VI-$P2Y_4$ Chimera	1215
$P2Y_4$-EP3.VI Chimera	1062

Table 9. Comprehensive List of Primer Sequences for Creation of Receptor Mutants and Chimeras

Primer Name	Primer Sequence[a]
EP4 Mutations	
EP4 Forward Primer	5' –AGG **GAA TTC** ATG *TAC CCA TAC GAC GTC CCA GAC TAC GCT* TCC ACT CCC GGG GTC AAT– 3'
EP4-t350 Reverse Primer	5' –GGA **GGA TCC** TAT TTA TTC AGC GGC AGA AGA GGC ATT TG– 3'
EP4-10ST Reverse Primer	5' –GGA **GGA TCC** TTA TAT ACA TTT TTC TGA TAA GTT CAG TGT– 3'
Receptor Chimeras	
EP3.VI/HA Tag Common Forward Primer	5' –GGA **GAA TTC** GCC GCC ACC ATG *TAC CCA TAC GAC GTC CCA GAC TAC GCT*– 3'
$P2Y_2$ Forward	5' –GGA **GAA TTC** GCC GCC ACC ATG *TAC CCA TAC GAC GTC CCA GAC TAC GCT* GCA GCA GAC CTG GGC CCC– 3'
$P2Y_2$ Reverse	5' –GGA **CTC GAG** CTA CAG CCG AAT GTC CTT AGT GTT CTC GCT– 3'
$P2Y_2$ Truncated Reverse	5'–CAC G**GG ATC C**AG GCA ACT GTT– 3'
$P2Y_2$ Tail Forward	5'–AGT TGC CT**G GAT CC**C GTC CTC TAC TTC CTG– 3'
$P2Y_4$ Forward	5' –GGA **GAA TTC** GCC GCC ACC ATG *TAC CCA TAC GAC GTC CCA GAC TAC GCT* GCC AGT ACA GAG TCC TCC CTG– 3'
$P2Y_4$ Reverse	5' –GGA **CTC GAG** TTA CAA TCT ATC TGC CCT AGG AGT AGA GCA– 3'

[a] Restriction site are noted in red (BamHI, GGATCC; EcoRI, GAATTC; XhoI, CTCGAG); underlining notes the Kozak sequence; the HA epitope tag sequence is noted in blue italics.

CHAPTER 3

LOCALIZATION OF HUMAN PGE_2 RECEPTORS IN MDCK CELLS

EP3 Isoforms

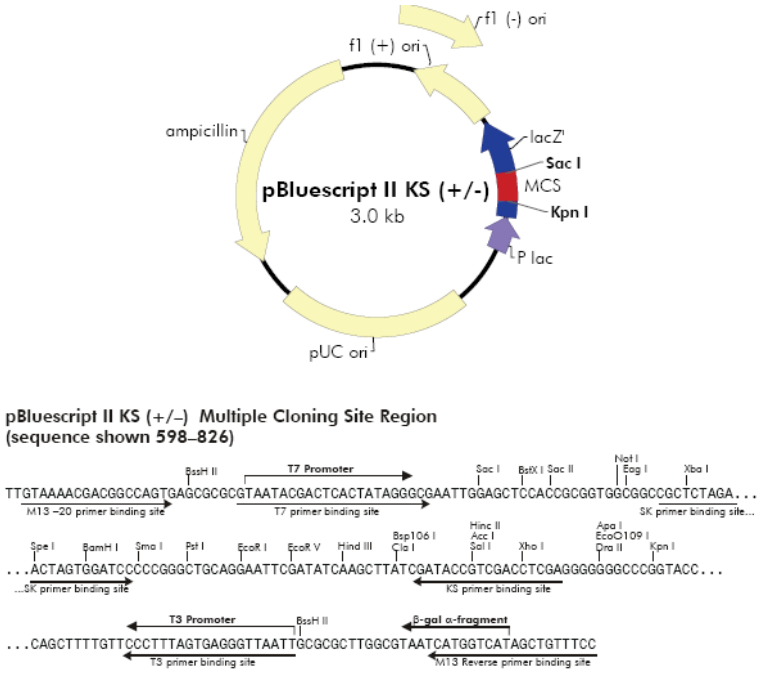
The epithelial Madin-Darby canine kidney (MDCK) cell line is commonly used in protein sorting studies. However, MDCK cells are notoriously difficult to transfect using standard methods, such as calcium phosphate or Lipofectamine 2000 mediated transfection. Moreover, because it takes at least five days for the cells to form compact monolayers, the transient expression of a desired gene is typically diminished by the time the cells are ready to be labeled with immunofluorescent antibodies. In order to combat these issues, we employed a retroviral vector system to stably express the human prostaglandin E_2 receptors in polarized epithelial cells.

Prostaglandin E_2 receptors are typically differentiated by their signal transduction pathways; however, there are additional characteristics that distinguish these receptors from one another. For example, previous studies from our laboratory have shown that the human prostaglandin E_2 receptors and isoforms display different agonist-induced internalization patterns (Desai et al., 2000; Bilson et al., 2004). The extent to which they internalize also differs among the receptors. Additionally, Hasegawa and colleagues (2000) showed the mouse EP3 isoforms display different membrane targeting in MDCK cells. The subcellular localization patterns for the human prostaglandin E_2 receptors have previously not been studied. We wished to further expand our studies and determine the membrane targeting of the human prostaglandin E_2 receptors in polarized epithelial cells.

To examine the subcellular targeting of the human PGE_2 receptors in MDCK cells, each HA-tagged receptor was first subcloned into the pBluescript II KS (+/-)

phagemid, a cloning vector with a phage origin, at the restriction sites EcoRI and HindIII **(Figure 6)**. Each receptor was then cloned into the pFB-Neo retroviral vector at the EcoRI and XhoI **(Figure 6)**. Products were obtained for EP2, EP3.I, EP3.II, EP3.VI, EP3.f and EP4. Each product was individually expressed in MDCK cells by retroviral transduction and stable cell lines were established. MDCK cells expressing each EP receptor were cultured in Corning Transwells and allowed to polarize for 7 days. The cells were then fixed and labeled with antibodies directed against the HA epitope tag and the epithelial tight junctions (ZO-I). The steady-state localization for each receptor in polarized monolayers was determined by confocal microscopy as described in CHAPTER 2.

A.



B.

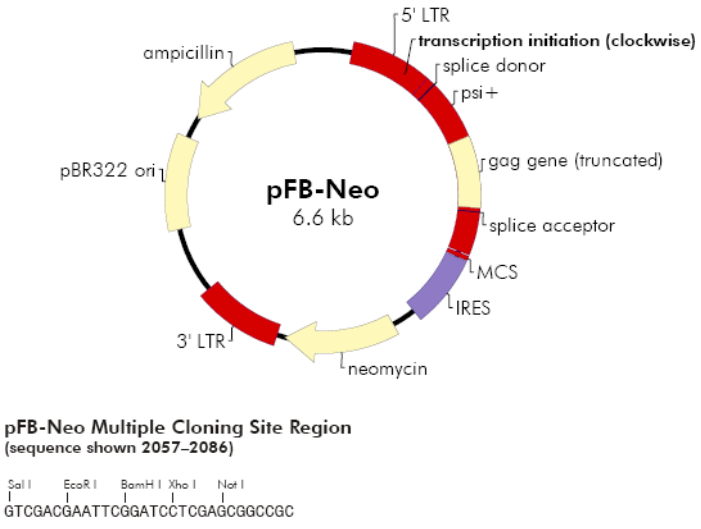


Figure 6. Schematic Representation of the pBluescript II KS (+/-) and pFB-Neo Cloning Vectors

Initially, we examined the subcellular localization of four different EP3 isoforms under basal conditions in polarized epithelial cells using confocal microscopy. **Figures 7** through **10** show *XY* and *XZ* cross sections of MDCK cells expressing the EP3 isoforms: I, II, VI or f. The top panels show sequential confocal scans parallel to the monolayer (*XY*), ranging from 1.2 to 1.8 microns. The bottom panel for each figure indicates a representative vertical cross section of the monolayer (*XZ*). Confocal micrographs of MDCK cells expressing the EP3 isoforms revealed that each isoform displays a unique subcellular targeting profile and that membrane targeting is not exclusive to one membrane domain. For control, MDCK cells were stained with secondary antibodies in the absence of primary anti-HA or anti-ZO antibodies. No staining was observed, indicating the primary antibodies were specific for the hemagglutinin (HA)-epitope tag or the zonula occludens (ZO), respectively.

Figure 7 shows the EP3.I isoform is distributed throughout the cell during steady-state. Staining is observed heavily at the basolateral domain and at the apical domain. The EP3.I receptor also appears within the intracellular compartment. **Figure 8** shows that the EP3.II isoform is localized heavily at the apical surface, with significant localization along the lateral regions of the cell. There is also a low lever of expression basal membrane and the intracellular compartment.

In contrast to the EP3.I and EP3.II isoforms, the EP3.VI isoform was expressed exclusively to the basolateral membrane **(Figure 9).** The EP3.f isoform was expressed heavily at the basolateral membrane of MDCK cells, with a low level of expression at the apical surface above the tight junctions **(Figure 10).** These results indicate that the EP3 isoforms display a unique and distinct pattern of polarized expression in MDCK cells.

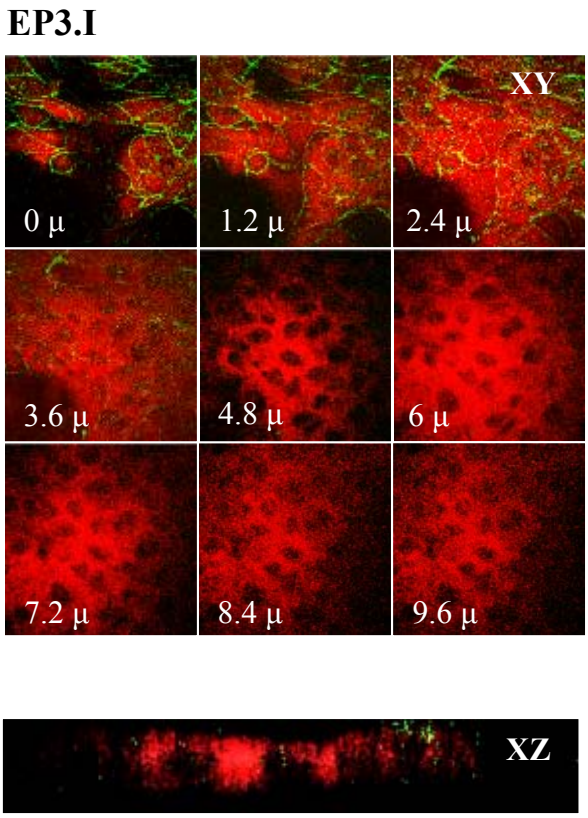


Figure 7. Confocal Microscopy Images of Madin-Darby Canine Kidney (MDCK) Cells Expressing the Hemagglutinin (HA)-tagged Human EP3.I Receptor

The upper panels indicate sequential confocal scans of 1.2 microns parallel to the monolayer (XY plane), while the lower panel shows a vertical cross section (XZ plane) of the cells. Red fluorescence represents HA-tagged receptors while green fluorescence represents the ZO-1 subunit of the tight junction complex. Staining of the human EP3.I receptor was observed at the apical and basolateral surface, as well as in the intracellular space of MDCK cells.

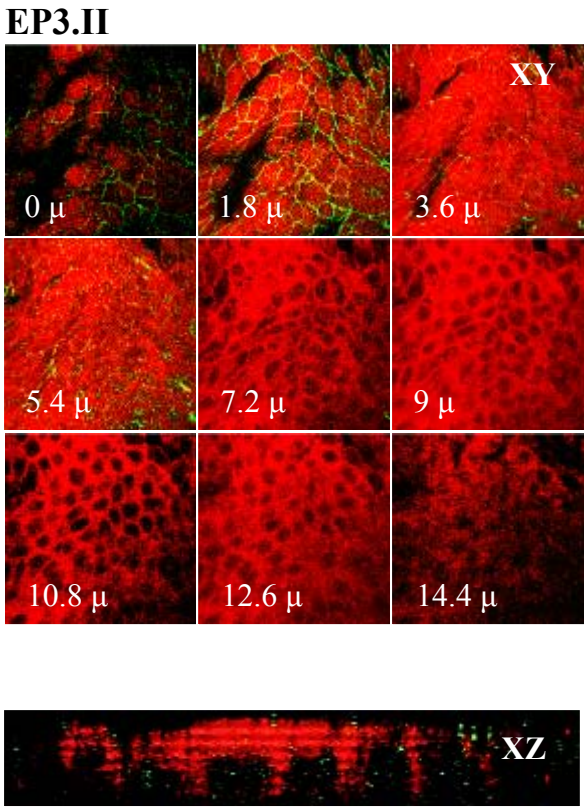


Figure 8. Confocal Microscopy Images of Madin-Darby Canine Kidney (MDCK) Cells Expressing the Hemagglutinin (HA)-tagged Human EP3.II Receptor

The upper panels indicate sequential confocal scans of 1.8 microns parallel to the monolayer (XY plane), while the lower panel shows a vertical cross section (XZ plane) of the cells. Red fluorescence represents HA-tagged receptors while green fluorescence represents the ZO-1 subunit of the tight junction complex. Staining of the human EP3.II receptor was observed at the apical and basolateral surface of MDCK cells.

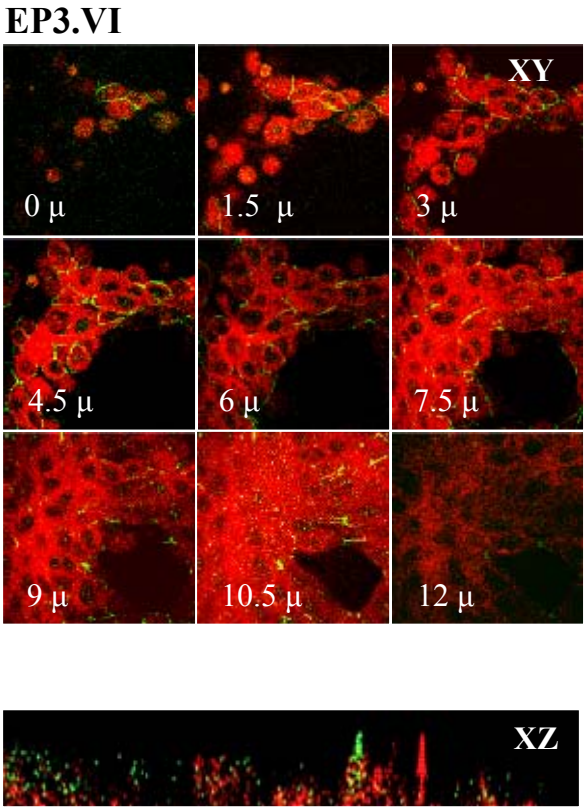


Figure 9. Confocal Microscopy Images of Madin-Darby Canine Kidney (MDCK) Cells Expressing the Hemagglutinin (HA)-tagged Human EP3.VI Receptor

The upper panels indicate sequential confocal scans of 1.5 microns parallel to the monolayer (XY plane), while the lower panel shows a vertical cross section (XZ plane) of the cells. Red fluorescence represents HA-tagged receptors while green fluorescence represents the ZO-1 subunit of the tight junction complex. Staining of the human EP3.VI receptor was observed exclusively at the basolateral surface of MDCK cells.

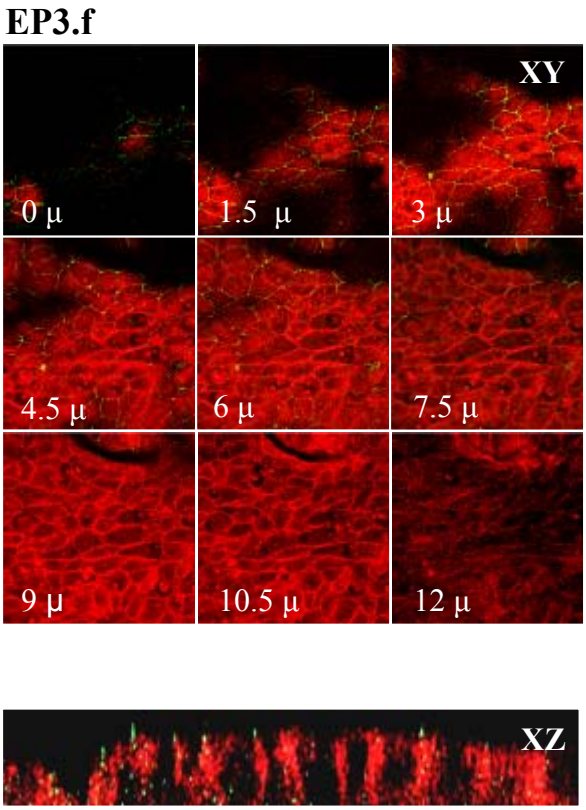


Figure 10. Confocal Microscopy Images of Madin-Darby Canine Kidney (MDCK) Cells Expressing the Hemagglutinin (HA)-tagged Human EP3.f Receptor

The upper panels indicate sequential confocal scans of 1.5 microns parallel to the monolayer (XY plane), while the lower panel shows a vertical cross section (XZ plane) of the cells. Red fluorescence represents HA-tagged receptors while green fluorescence represents the ZO-1 subunit of the tight junction complex. Staining of the human EP3.f receptor was observed predominantly at the basolateral surface, with a low level of expression at the apical surface of MDCK cells.

EP2 and EP4 Receptors

Previously, our laboratory established the agonist-induced internalization patterns for these receptors, indicating that while the EP4 receptor internalized rapidly, the EP2 receptor did not internalize at all (Desai et al., 2000). As a way to further characterize these receptors, we looked at the subcellular localization patterns of the human EP2 and EP4 receptors in MDCK cells under basal conditions.

As described above, **Figure 11** and **Figure 12** show *XY* and *XZ* cross sections of MDCK cells expressing the EP2 and EP4 receptors, respectively. The top panels show sequential confocal scans parallel to the monolayer (*XY*), ranging from 1.0 to 1.5 microns. The bottom panel for each figure indicates a representative vertical cross section of the monolayer (*XZ*). Confocal micrographs of MDCK cells individually expressing these receptors revealed that these receptors display similar patterns of localization.

Figure 11 shows the EP2 receptor is localized to the basolateral domain during steady-state. Staining is observed heavily at the lateral surface and to a lesser degree at the basal membrane. **Figure 12** shows that the EP4 receptor is also localized heavily at the basolateral domain, with significant localization along the lateral regions of the cell. These studies show that these receptors, which both couple to the G_s pathway yet display different internalization patterns, display the same localization patterns in MDCK cells.

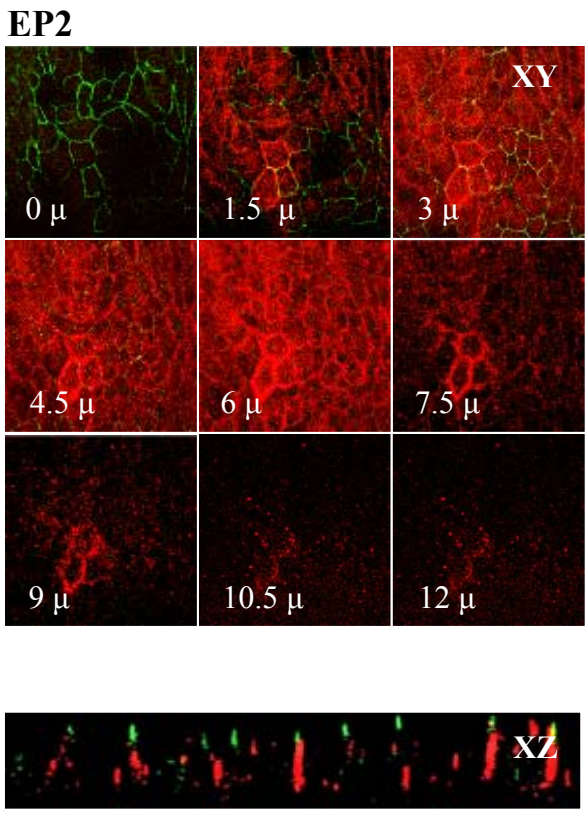


Figure 11. Confocal Microscopy Images of Madin-Darby Canine Kidney (MDCK) Cells Expressing the Hemagglutinin (HA)-tagged Human EP2 Receptor

The upper panels indicate sequential confocal scans of 1.5 microns parallel to the monolayer (XY plane), while the lower panel shows a vertical cross section (XZ plane) of the cells. Red fluorescence represents HA-tagged receptors while green fluorescence represents the ZO-1 subunit of the tight junction complex. Staining of the human EP2 receptor was observed exclusively at the basolateral surface of MDCK cells.

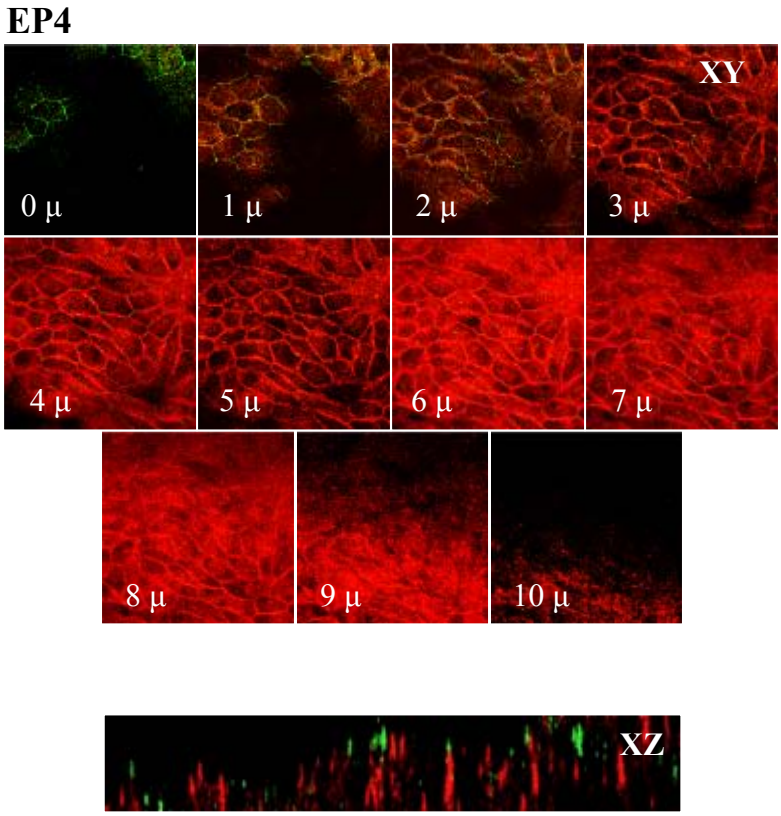


Figure 12. Confocal Microscopy Images of Madin-Darby Canine Kidney (MDCK) Cells Expressing the Hemagglutinin (HA)-tagged Human EP4 Receptor

The upper panels indicate sequential confocal scans of 1 micron parallel to the monolayer (XY plane), while the lower panel shows a vertical cross section (XZ plane) of the cells. Red fluorescence represents HA-tagged receptors while green fluorescence represents the ZO-1 subunit of the tight junction complex. Staining of the human EP4 receptor was observed exclusively at the basolateral surface of MDCK cells.

CHAPTER 4

EFFECTS OF C-TAIL MUTATIONS AND RECEPTOR CHIMERAS ON THE LOCALIZATION PATTERNS OF PGE_2 RECEPTORS IN MDCK CELLS

hEP3.I-5A Mutant

In previous work, we prepared several mutants within the C-tail of the EP3.I receptor isoform to determine the role of the SSSTS (residues 369-373) amino acid sequence on the internalization of the receptor **(Figure 13)**. Serine and threonine residues are potential sites of phosphorylation and may play a role in regulation of receptor function in response to agonist stimulation. Using confocal microscopy, it was determined that the various mutants displayed different internalization patterns. Most notably, the complete 5A mutant did not internalize following continued exposure to PGE_2.

We wished to determine whether the change in receptor internalization of the hEP3.I-5A mutant reflected differences in basal-level localization patterns of the receptor in MDCK cells. In order to express the hEP3.I-5A mutant in MDCK cells, we created retroviral constructs of the HA-epitope tagged mutant and the pFB-neo retroviral vector. Using antibiotic selection, stable MDCK cell lines expressing the hEP3.I-5A mutant were established and cultured in Corning Transwells for 7 days to allow for cell polarization. The cells were then fixed and labeled with antibodies directed against the HA epitope tag and the epithelial tight junctions (ZO-I). The steady-state localization for the receptor mutant in polarized monolayers was determined by confocal microscopy. The SSSTS did not appear to have any effect on the localization pattern of the EP3.I isoform at basal

levels **(Figure 14)**. The hEP3.I-5A mutant did not localize to a specific surface as it was evenly distributed to the apical and basolateral domains, as well as within the intracellular compartment. The results of this study fully correspond to the distribution pattern observed for the hEP3.I wild type receptor **(Figure 6)**, suggesting that serine and threonine residues located within the C-tail of the human EP3.I isoform do not play a role in targeting the receptor during steady-state.

EP3.I C-tail WT	IRYHTNNYA**SSSTS**LPCQCSSTLMWSDHLER*

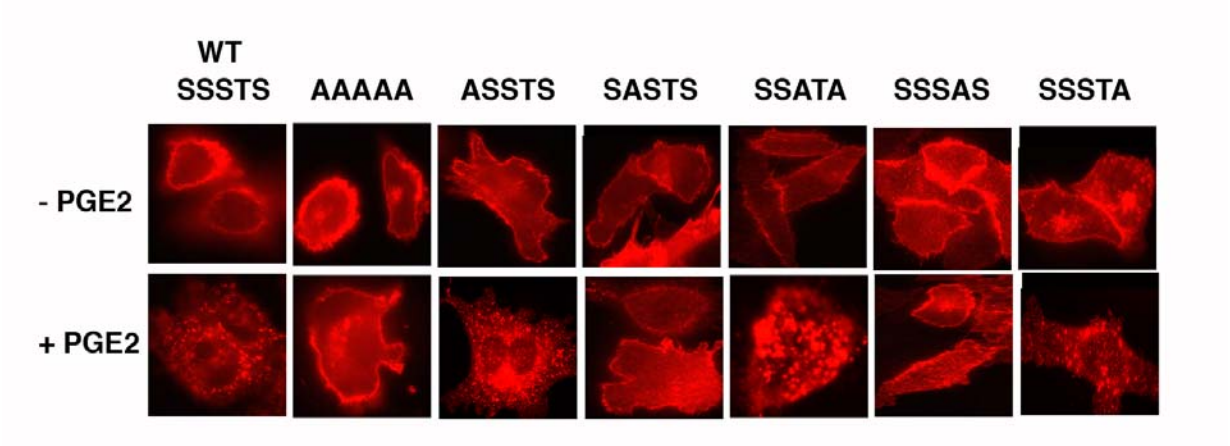


Figure 13. Internalization Patterns of hEP3.I C-tail Mutants in HEK293 Cells

EP3.I C-tail mutations were created using site-directed mutagenesis to substitute serine and/or threonine to alanine. HEK293 cells were then transfected with HA-EP3.I C-tail mutants and images were obtained using a Leica confocal microscope.

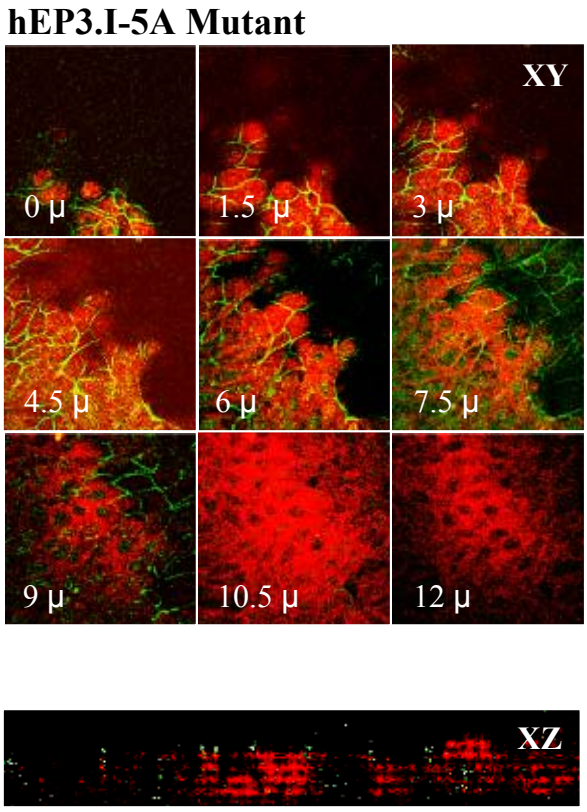


Figure 14. Confocal Microscopy Images of Madin-Darby Canine Kidney (MDCK) Cells Expressing the Hemagglutinin (HA)-tagged Human EP3.I-5A Mutant Receptor

The upper panels indicate sequential confocal scans of 1.5 microns parallel to the monolayer (XY plane), while the lower panel shows a vertical cross section (XZ plane) of the cells. Red fluorescence represents HA-tagged receptors while green fluorescence represents the ZO-1 subunit of the tight junction complex. Staining of the human EP3.I-5A receptor was observed at the apical and basolateral surface of MDCK cells.

hEP4-10ST and hEP4-t350 Mutants

We then wanted to determine if the C-tail region of the EP4 receptor plays a role in receptor localization. Previously, our laboratory created EP4 receptor mutants to demonstrate the role of the C-tail in agonist-induced desensitization and internalization. We have shown that the EP4 C-terminal domain is essential for agonist-induced desensitization and identified a 14-amino-acid sequence containing six serine residues that is involved in short-term desensitization of the EP4 receptor (Bastepe and Ashby, 1997; Bastepe and Ashby, 1999). Additional studies established that the EP4 C-tail is involved in receptor internalization; however, serine and threonine residues were not involved (Desai et al., 2000).

For these studies we utilized two human EP4 receptor mutants **(Figure 5)**. In one mutation, the receptor C-tail was truncated at amino acid 350 (hEP4-t350 mutant). The other receptor mutant consisted of a ten amino acid substitution of serines and threonines in the C-tail between amino acids 354 and 378 to alanine (hEP4-10ST mutant). The specific amino acid substitutions are as follows: S354A, S359A, S364A, T369A, S370A, S371A, S374A, S377A, S379A, and S382A. Retroviral constructs of the HA-epitope tagged EP4 mutants and the pFB-neo retroviral vector were constructed. Using antibiotic selection, stable MDCK cell lines expressing the hEP4-10ST or hEP4-t350 mutant were established and cultured in Corning Transwells for 7 days to allow for cell polarization. The cells were then fixed and labeled with antibodies directed against the HA epitope tag and the epithelial tight junctions (ZO-I). The steady-state localization for the receptor mutant in polarized monolayers was determined by confocal microscopy.

Analysis of the confocal microscopy images revealed that both the hEP4-10ST and hEP4-t350 receptors localized predominantly to the basolateral surface of MDCK cells **(Figures 15 and 16)**. Both receptors may also be contained within the intracellular space; however, this is observation is weak as there is no visible nucleus. This suggests that the staining pattern observed in the XZ plane is due to imaging parallel to the lateral cell surface. With this taken into account, the hEP4-10ST and hEP4-t350 receptor mutants showed similar localization patterns as the EP4 wild type **(Figure 12).** This indicates that the C-tail of the EP4 receptor does not play a role in basal level localization of the receptor and further confirms that serine and threonine residues have no effect on subcellular localization.

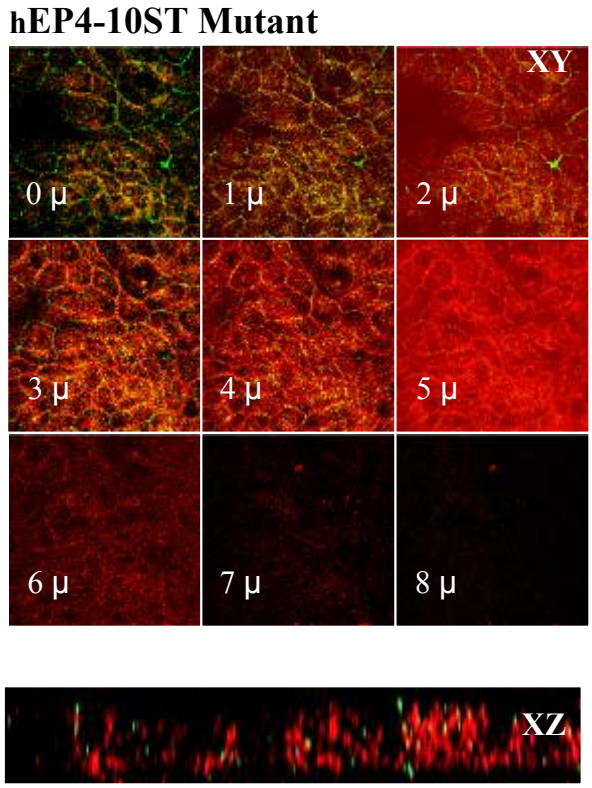


Figure 15. Confocal Microscopy Images of Madin-Darby Canine Kidney (MDCK) Cells Expressing the Hemagglutinin (HA)-tagged Human EP4-10ST Mutant Receptor

The upper panels indicate sequential confocal scans of 1 micron parallel to the monolayer (XY plane), while the lower panel shows a vertical cross section (XZ plane) of the cells. Red fluorescence represents HA-tagged receptors while green fluorescence represents the ZO-1 subunit of the tight junction complex. Staining of the human EP4-10ST receptor was observed predominantly at the basolateral surface of MDCK cells.

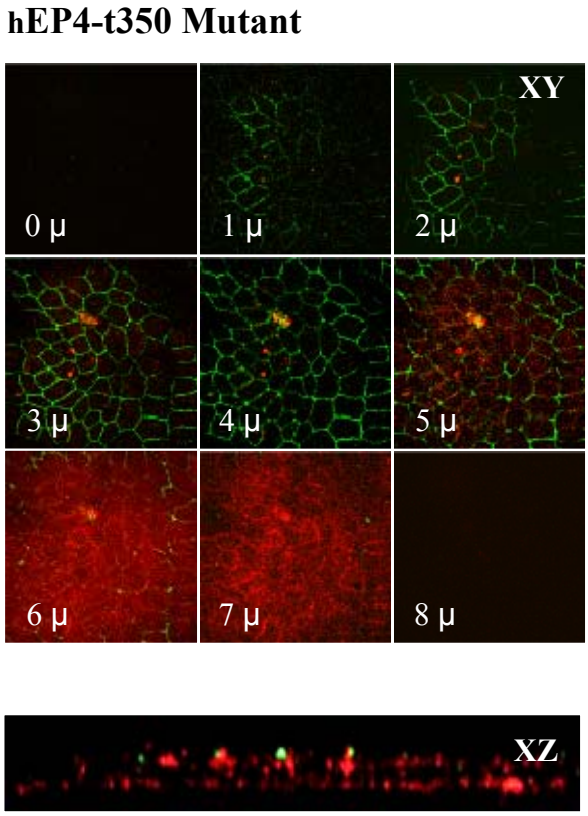


Figure 16. Confocal Microscopy Images of Madin-Darby Canine Kidney (MDCK) Cells Expressing the Hemagglutinin (HA)-tagged Human EP4-t350 Mutant Receptor

The upper panels indicate sequential confocal scans of 1 microns parallel to the monolayer (XY plane), while the lower panel shows a vertical cross section (XZ plane) of the cells. Red fluorescence represents HA-tagged receptors while green fluorescence represents the ZO-1 subunit of the tight junction complex. Staining of the human EP4-t350 receptor was observed predominantly at the basolateral surface of MDCK cells.

Purinergic $P2Y_2$ and $P2Y_4$ Receptors

Many studies have exploited the chimeric receptor approach between similar proteins to elucidate the signals and mechanisms that govern GPCR targeting (Saunders et al., 1998; Nadler et al., 2001; Qi et al., 2005). We chose to make receptor chimeras utilizing two purinergic receptor subtypes, $P2Y_2$ and $P2Y_4$. These are G protein-coupled receptors, which both couple to the G_q pathway and localize to the apical surface of MDCK cells. Through chimera and mutational analysis, Qi and colleagues (2005) determined that the sorting signal for the $P2Y_2$ receptor was located within its first extracellular loop, while the sorting signal for the $P2Y_4$ receptor was located within its C-tail. We first expressed the $P2Y_2$ and $P2Y_4$ receptors in MDCK cells using retroviral transduction and observed their localization patterns using confocal microscopy. In accordance with the results published by Wolf et al., 2004, the $P2Y_2$ and $P2Y_4$ receptors both localized to the apical surface in MDCK cells **(Figure 17)**.

$P2Y_2$ Receptor **$P2Y_4$ Receptor**

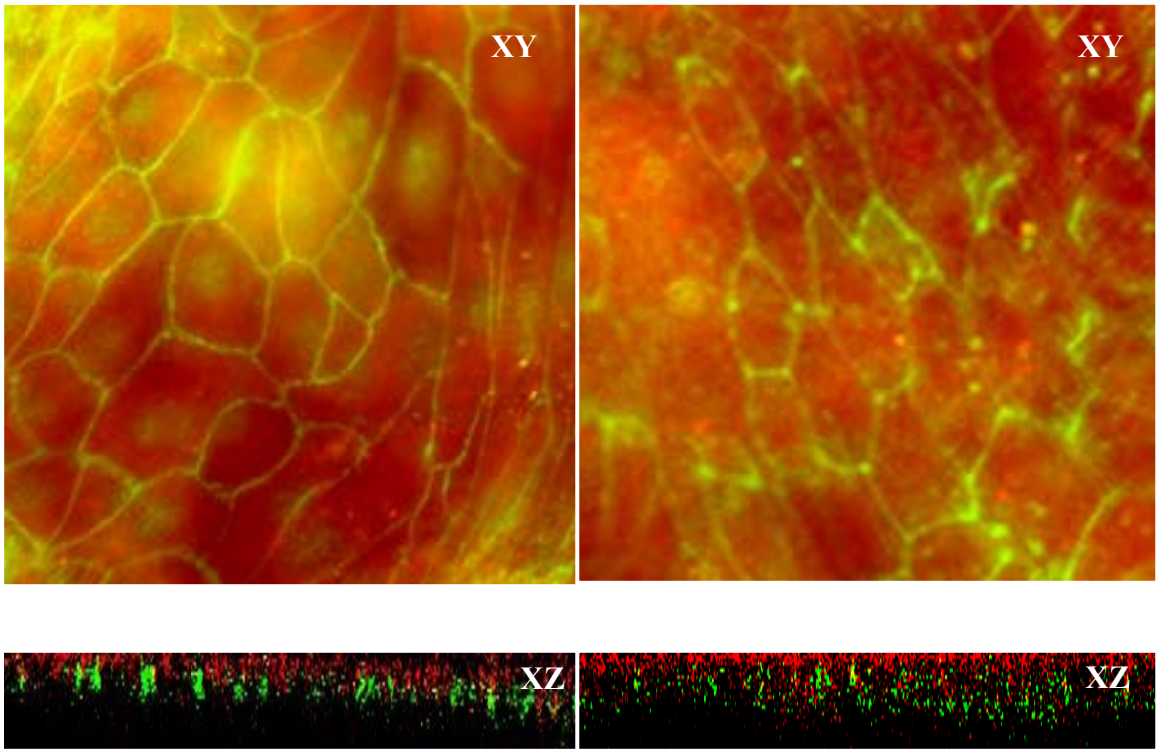


Figure 17. Immunofluorescence and Confocal Microscopy Images of Madin-Darby Canine Kidney (MDCK) Cells Expressing the Hemagglutinin (HA)-tagged Purinergic $P2Y_2$ and $P2Y_4$ Receptors

The upper panels indicate representative immunofluorescence images of the monolayer (XY plane), while the lower panel shows a vertical cross section (XZ plane) of the cells obtained by confocal microscopy. Red fluorescence represents HA-tagged receptors while green fluorescence represents the ZO-1 subunit of the tight junction complex. Both $P2Y_2$ and $P2Y_4$ receptors were expressed exclusively at the apical surface of MDCK cells.

EP3.VI and $P2Y_4$ Chimeras

Proteins are sorted to specific membrane domains based on signals located within their primary sequence, which are recognized by the cell. To examine the mechanism of membrane targeting in prostaglandin receptors, we first chose to utilize the human EP3.VI isoform, which localizes to the basolateral surface of MDCK cells, and the human $P2Y_4$ receptor, which localizes to the apical surface of MDCK cells **(Figure 18)**. We have previously shown that the EP3 isoforms demonstrate unique patterns of subcellular localization in polarized epithelial cells, suggesting their C-terminal domains contain sorting signals, as the receptors are homologous over the first 359 amino acids of their sequences. In 2005, Qi and colleagues first reported that the $P2Y_4$ receptor achieves apical sorting by a signal located within its C-tail. We hypothesized that each chimera would localize to the proper cell surface dictated by the C-tail region.

Both receptors contain an internal BamHI restriction site within their C-terminal tail, which we were able to exploit in order to create two receptor chimeras, EP3.VI-$P2Y_4$ and $P2Y_4$-EP3.VI. Each parent receptor was first cloned into the pFB-neo retroviral vector. The C-tails were then cut from each plasmid using restriction enzymes and ligated into the respective chimera plasmid. Each construct was stably expressed in MDCK cells and polarized on transwell plates. The cells were then fixed and labeled with antibodies directed against the HA epitope tag and the epithelial tight junctions (ZO-I). The steady-state localization for each receptor chimera was determined by confocal microscopy. The top panels in **Figures 19** and **20** display representative fluorescence images of the chimeras, while the bottom panels reveal the confocal XZ scan, perpendicular to the monolayer. Interestingly, both receptor chimeras displayed non-

specific localization patterns in the MDCK cell system. The EP3.VI-$P2Y_4$ and $P2Y_4$-EP3.VI chimeras both localized to the apical and basolateral surfaces, in addition to showing some localization to the intracellular space.

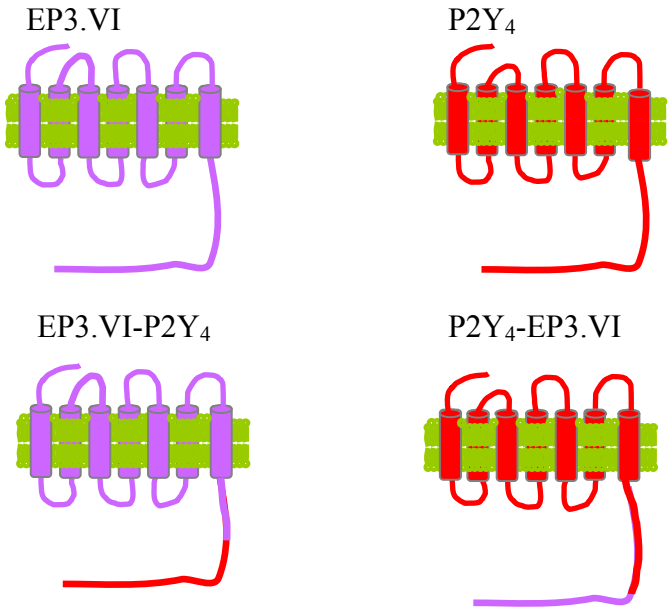


Figure 18. Schematic Representation of EP3.VI and $P2Y_4$ Receptor Chimeras

The human EP3.VI and $P2Y_4$ receptors were used as the parent constructs from which the EP3.VI-$P2Y_4$ and $P2Y_4$-EP3.VI chimeras were derived. The length of the EP3.VI C-tail was 52 amino acids in length, while the $P2Y_4$ receptor C-tail was 64 amino acids in length.

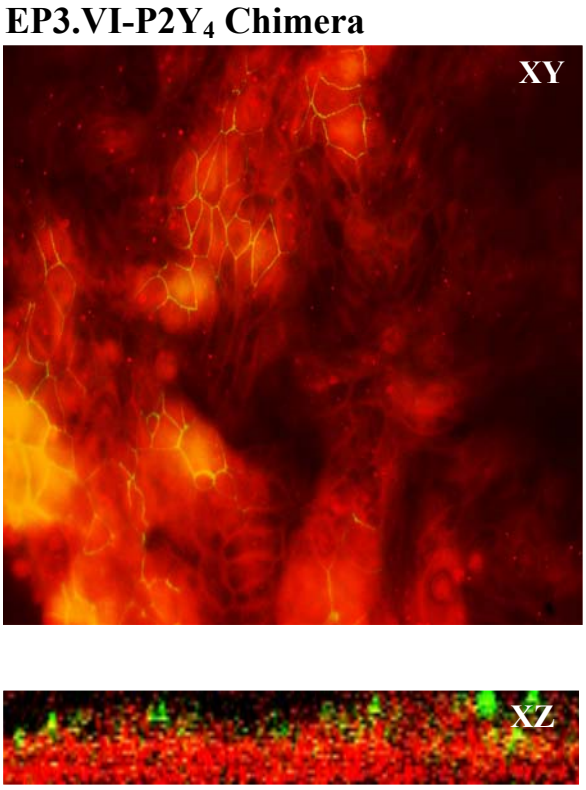


Figure 19. Immunofluorescence and Confocal Microscopy Images of Madin-Darby Canine Kidney (MDCK) Cells Expressing the Hemagglutinin (HA)-tagged EP3.VI- $P2Y_4$ Receptor Chimeras

The upper panel indicates a representative immunofluorescence image of the monolayer (XY plane), while the lower panel shows a vertical cross section (XZ plane) of the cells obtained by confocal microscopy. Red fluorescence represents HA-tagged receptors while green fluorescence represents the ZO-1 subunit of the tight junction complex. Staining of EP3.VI-$P2Y_4$ receptors was observed throughout MDCK cells.

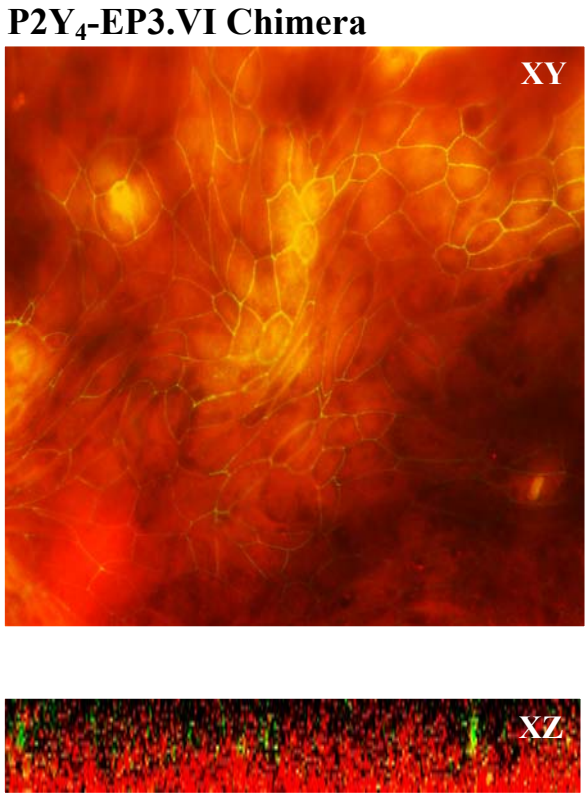


Figure 20. Immunofluorescence and Confocal Microscopy Images of Madin-Darby Canine Kidney (MDCK) Cells Expressing the Hemagglutinin (HA)-tagged $P2Y_4$- EP3.VI Receptor Chimeras

The upper panel indicates a representative immunofluorescence image of the monolayer (XY plane), while the lower panel shows a vertical cross section (XZ plane) of the cells obtained by confocal microscopy. Red fluorescence represents HA-tagged receptors while green fluorescence represents the ZO-1 subunit of the tight junction complex. Staining of $P2Y_4$- EP3.VI receptors was observed throughout MDCK cells.

EP3.VI and $P2Y_2$ Chimeras

We also wanted to examine the subcellular targeting of chimeras constructed using the human EP3.VI and $P2Y_2$ receptors. As previously mentioned, the purinergic $P2Y_2$ receptor contains a sorting signal for apical localization within its first extracellular loop. For this experiment, the EP3.VI-$P2Y_2$ chimera would contain no known sorting signals, while the $P2Y_2$-EP3.VI chimera would contain two potential sorting signals in the first extracellular loop and C-terminal domain, respectively **(Figure 21)**.

The BamHI restriction site within the C-tail of the EP3.VI isoform was again used to create the chimeras; however, the $P2Y_2$ receptor does not contain this site within its C-tail sequence. The BamHI restriction site was inserted into the C-tail of the $P2Y_2$ receptor at amino acids 302/303 using custom designed PCR primers. Each parent receptor was then cloned into the pFB-neo retroviral vector. The C-tails were cut from each plasmid using restriction enzymes BamHI and XhoI and ligated into the respective chimera plasmid. Each construct was stably expressed in MDCK cells and polarized on transwell plates. The cells were then fixed and labeled with antibodies directed against the HA epitope tag and the epithelial tight junctions (ZO-I). The steady-state localization for each receptor chimera was determined by confocal microscopy. The top panels in **Figures 22** and **23** display representative fluorescence images of the chimeras, while the bottom panels reveal the confocal XZ scan, perpendicular to the monolayer.

Interestingly, the EP3.VI-$P2Y_2$ chimera, which contains no known sorting signals, localized to the apical surface of MDCK cells **(Figure 22)**. The $P2Y_2$-EP3.VI chimera, which contains the purinergic $P2Y_2$ apical sorting signal in the first extracellular loop and

the presumed EP3.VI sorting signal in the C-tail, localized to the basolateral domain of MDCK cells **(Figure 23)**. These results confirm that the C-tail region of the EP3.VI receptor does contain a sorting signal within its sequence that directs the isoform to the basolateral surface of polarized epithelial cells. It also suggests that the apical sorting signal is weaker than, or completely blocked by the basolateral sorting signal within the EP3.VI C-tail.

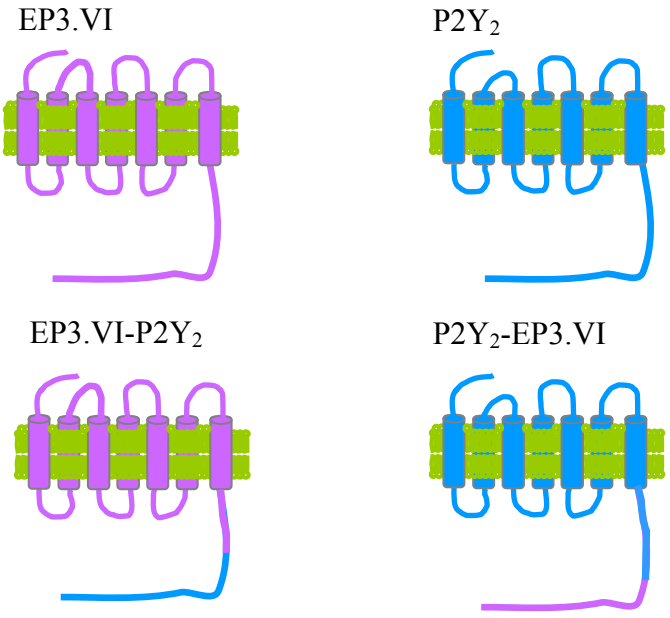


Figure 21. Schematic Representation of EP3.VI and $P2Y_2$ Receptor Chimeras

The human EP3.VI and $P2Y_2$ receptors were used as the parent constructs from which the EP3.VI-$P2Y_2$ and $P2Y_2$-EP3.VI chimeras were derived. The length of the EP3.VI C-tail was 52 amino acids in length, while the $P2Y_2$ receptor C-tail was 75 amino acids in length.

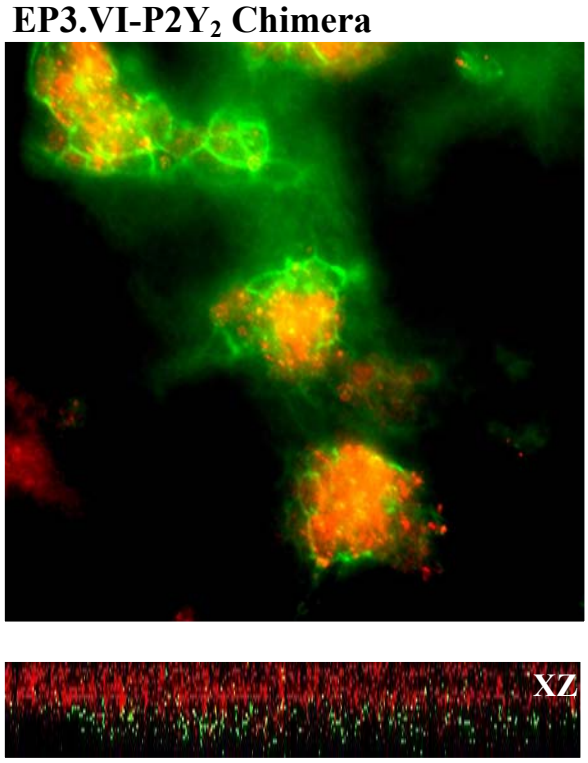


Figure 22. Immunofluorescence and Confocal Microscopy Images of Madin-Darby Canine Kidney (MDCK) Cells Expressing the Hemagglutinin (HA)-tagged EP3.VI-P2Y$_2$ Receptor Chimeras

The upper panel indicates a representative immunofluorescence image of the monolayer (XY plane), while the lower panel shows a vertical cross section (XZ plane) of the cells obtained by confocal microscopy. Red fluorescence represents HA-tagged receptors while green fluorescence represents the ZO-1 subunit of the tight junction complex. Staining of EP3.VI- P2Y$_2$ receptors was observed exclusively at the apical surface of MDCK cells.

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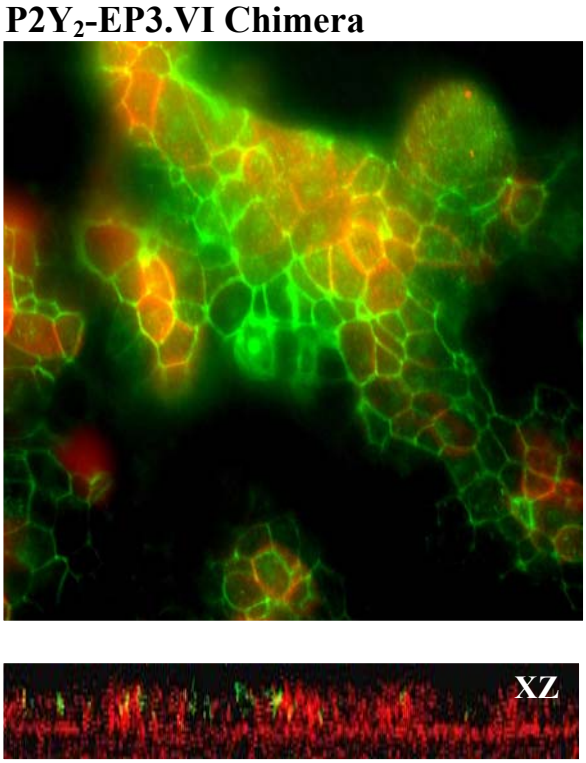

Figure 23. Immunofluorescence and Confocal Microscopy Images of Madin-Darby Canine Kidney (MDCK) Cells Expressing the Hemagglutinin (HA)-tagged $P2Y_2$- EP3.VI Receptor Chimeras

The upper panel indicates a representative immunofluorescence image of the monolayer (XY plane), while the lower panel shows a vertical cross section (XZ plane) of the cells obtained by confocal microscopy. Red fluorescence represents HA-tagged receptors while green fluorescence represents the ZO-1 subunit of the tight junction complex. Staining of $P2Y_2$-EP3.VI receptors was observed exclusively at the basolateral surface of MDCK cells.

CHAPTER 5

LOCALIZATION OF HUMAN PGE_2 RECEPTORS AND MUTANTS IN BEAS-2B CELLS

To determine whether subcellular localization of the human prostaglandin E_2 receptors in polarized epithelial cells was a cell-specific phenomenon, we expressed various HA-tagged receptors in human lung epithelial cells, or BEAS-2B cells. The airway-derived BEAS-2B cell line is commonly used as an *in vitro* model for the airway epithelium, specifically serving to study drug metabolism (Forbes, 2000). BEAS-2B cells typically do not form tight junctions (Noah et al., 1995; Lopez-Souza et al., 2004), which proved to be a limiting factor for confocal microscopy as we were unable to rely on labeling the tight junctions with an antibody to serve as a marker. Additionally, BEAS-2B cells appeared to form thinner monolayers compared to MDCK cells, making it unfeasible to obtain XZ scans of the cells.

Human EP3.I Wild Type and EP3.I-5A Mutant

We first expressed the human EP3.I wild type isoform and the human EP3.I-5A mutant in BEAS-2B cells using retroviral transfection and created stable cell lines using antibiotic selection. BEAS-2B cells were seeded onto Corning Transwell plates and allowed to polarize for 7 days. The cells were then fixed and labeled with antibodies directed against the HA epitope tag. Analysis of the steady-state localization for the receptor mutant in polarized monolayers was determined by sequential XY scans using a Leica TCS-NT confocal microscope.

The results from **Figure 24** show that the human EP3.I wild type isoform localized to the apical surface, marked by the fluorescence of the receptor covering the "top" of the cell surface. The EP3.I wild type isoform also localized to the intracellular space, which is observed as fluorescence of the receptor throughout the cell in the descending layers. The results from **Figure 25** show the human EP3.I-5A mutant localized to the basolateral surface, marked by the distinct "ring" staining around the cell membrane. The hEP3.I-5A mutant also appeared to display staining at the apical surface and intracellular compartment, but at lower levels than the basolateral surface. These results are comparable to the studies previously shown of these receptors in MDCK cells.

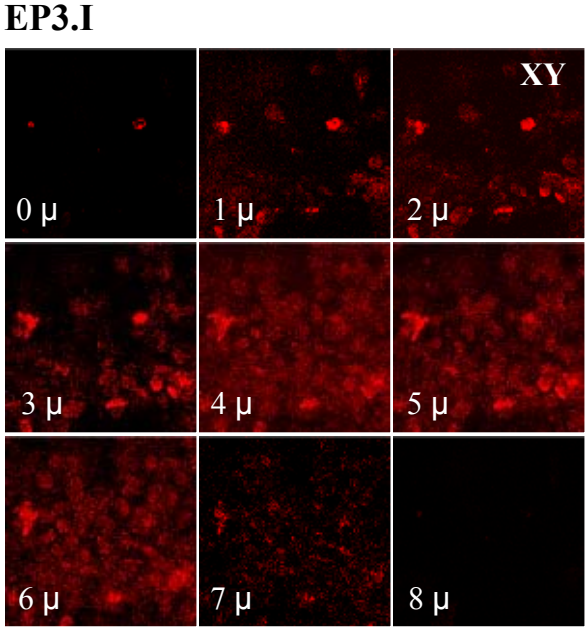

Figure 24. Confocal Microscopy Images of human bronchial epithelial (BEAS-2B) Cells Expressing the Hemagglutinin (HA)-tagged Human EP3.I Receptor

The panels indicate sequential confocal scans of 1 micron parallel to the monolayer (XY plane). Red fluorescence represents HA-tagged receptors. Staining of the human EP3.I receptor was observed predominantly at the apical surface of BEAS-2B cells, with additional expression observed in the intracellular space.

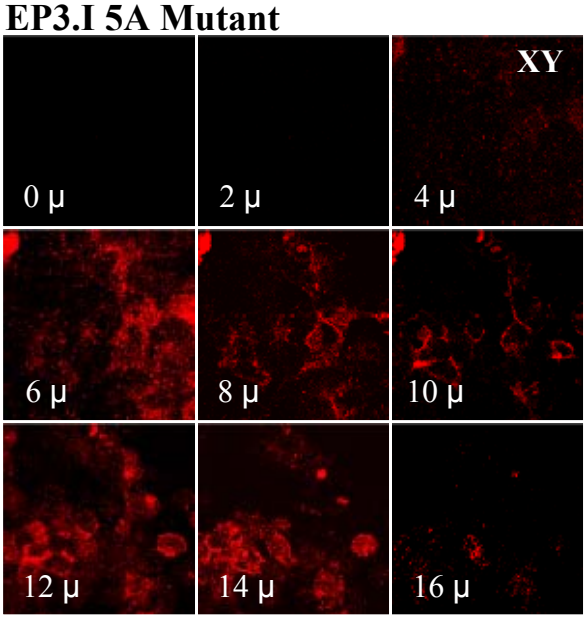

Figure 25. Confocal Microscopy Images of human bronchial epithelial (BEAS-2B) Cells Expressing the Hemagglutinin (HA)-tagged Human EP3.I 5A Mutant Receptor

The panels indicate sequential confocal scans of 2 microns parallel to the monolayer (XY plane). Red fluorescence represents HA-tagged receptors. Staining of the human EP3.I-5A receptor was observed at the basolateral surface of BEAS-2B cells, with lower expression observed at the apical surface and in the intracellular space.

EP2 and EP4 Receptors

We have previously shown that the human EP2 and EP4 receptors, which both couple to the G_s pathway, are both targeted to the basolateral surface of Madin-Darby canine kidney cells. To determine whether their targeting profile was dependent on cell type, we chose to examine the subcellular targeting of the human EP2 and EP4 receptors in BEAS-2B cells. Each receptor was cloned into the pFB-neo retroviral vector and expressed in the BEAS-2B cell line using retroviral transfection methods. Stable cell lines were established using antibiotic selection. BEAS-2B cells were seeded onto Corning Transwell plates and allowed to polarize for 7 days. The cells were then fixed and labeled with antibodies directed against the HA epitope tag. Analysis of the steady-state localization for the receptor mutant in polarized monolayers was determined by sequential XY scans using a Leica TCS-NT confocal microscope.

The results in **Figure 26** show that human EP2 receptor localizes strongly to the basolateral surface of BEAS-2B cells, with staining at the apical surface observed in a small number cells. **Figure 27** shows the results obtained for the human EP4 receptor, which localized only to the basolateral surface. These results were very similar to those obtained in MDCK cells, where both receptors localized to the basolateral surface. This further supports our earlier observation that subcellular targeting is independent of cell type.

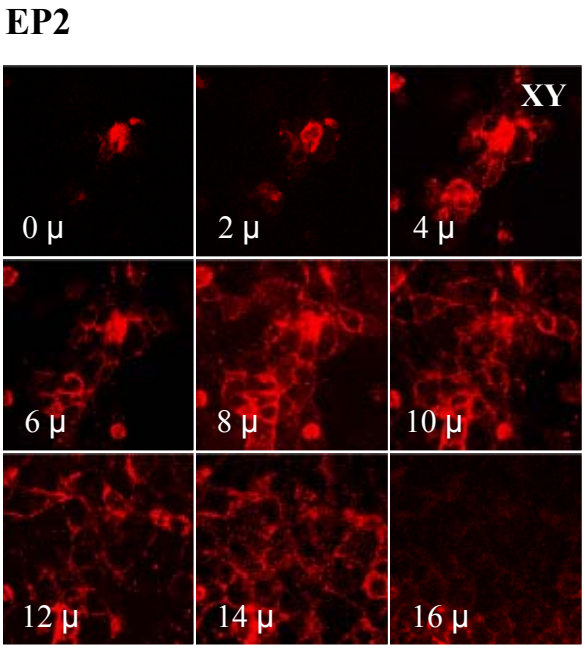

Figure 26. Confocal Microscopy Images of human bronchial epithelial (BEAS-2B) Cells Expressing the Hemagglutinin (HA)-tagged Human EP2 Receptor

The panels indicate sequential confocal scans of 2 microns parallel to the monolayer (XY plane). Red fluorescence represents HA-tagged receptors. Staining of the human EP2 receptor was observed predominantly at the basolateral surface of BEAS-2B cells, with minor expression observed at the apical surface.

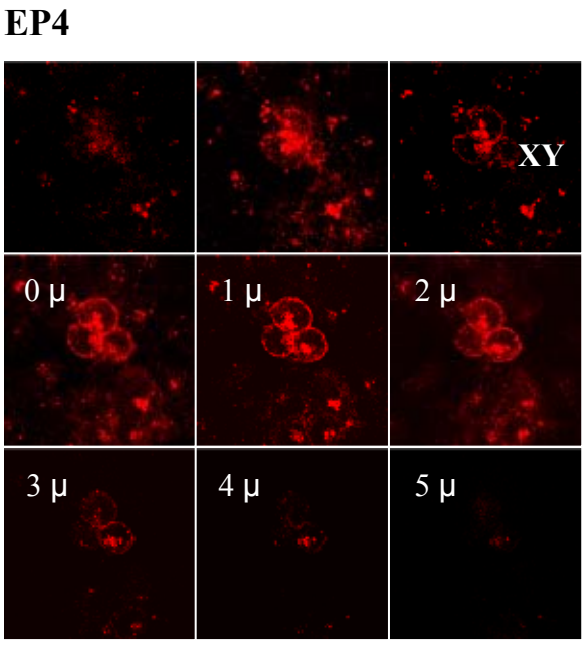

Figure 27. Confocal Microscopy Images of human bronchial epithelial (BEAS-2B) Cells Expressing the Hemagglutinin (HA)-tagged Human EP4 Receptor

The panels indicate sequential confocal scans of 2 microns parallel to the monolayer (XY plane). Red fluorescence represents HA-tagged receptors. Staining of the human EP4 receptor was observed exclusively at the basolateral surface of BEAS-2B cells.

hEP4-10ST and hEP4-t350 Mutants

We previously determined that these mutations did not alter the targeting pattern of the human EP4 receptor in Madin-Darby canine kidney cells (CHAPTER 4). To further complete this study, we expressed the two human EP4 receptor mutants in BEAS-2B cells to determine if these specific C-tail mutations would alter the subcellular localization patterns in this model. As previously described, each receptor mutant was cloned into the pFB-neo retroviral vector and expressed in the BEAS-2B cell line using retroviral transfection methods. Stable cell lines were established using antibiotic selection. BEAS-2B cells were seeded onto Corning Transwell plates and allowed to polarize for 7 days. The cells were then fixed and labeled with antibodies directed against the HA epitope tag. Analysis of the steady-state localization for the receptor mutant in polarized monolayers was determined by sequential XY scans using a Leica TCS-NT confocal microscope.

Under basal conditions, the human EP4-10ST receptor localized to the basolateral surface of BEAS-2B cells, marked by the distinct ring-like staining around the edge of the cell membrane **(Figure 28)**. In MDCK cells, the hEP4-10ST mutant localized strongly to the basolateral surface with a low level of intracellular staining observed. **Figure 29** shows the results of the human EP4-t350 receptor mutant. In BEAS-2B cells, this receptor localized to the basolateral surface with some intracellular staining observed. In MDCK cells, this mutant displayed the same pattern of staining. These results again confirm that receptor targeting is independent of cell type.

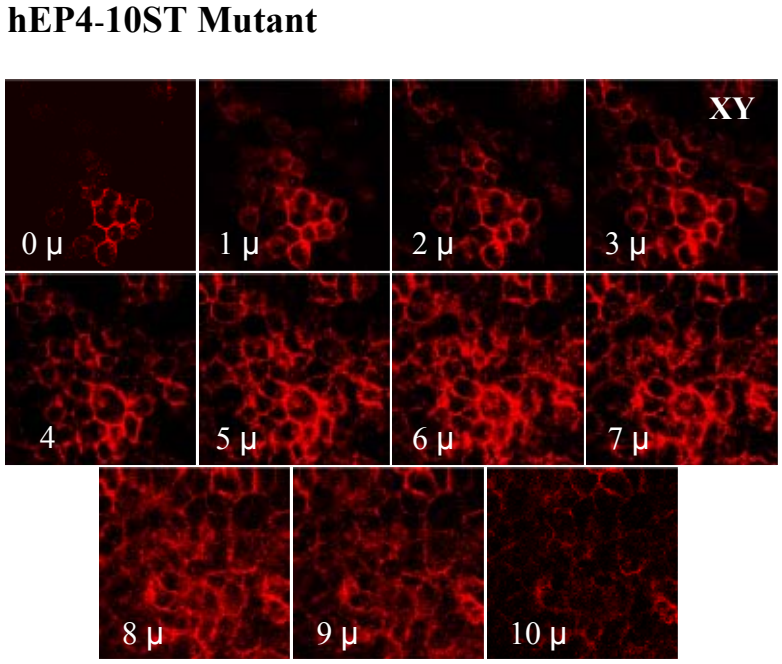

Figure 28. Confocal Microscopy Images of human bronchial epithelial (BEAS-2B) Cells Expressing the Hemagglutinin (HA)-tagged Human EP4-10ST Mutant Receptor

The panels indicate sequential confocal scans of 1 micron parallel to the monolayer (XY plane). Red fluorescence represents HA-tagged receptors. Staining of the human EP4-10ST receptor was observed predominantly at the basolateral surface of BEAS-2B cells.

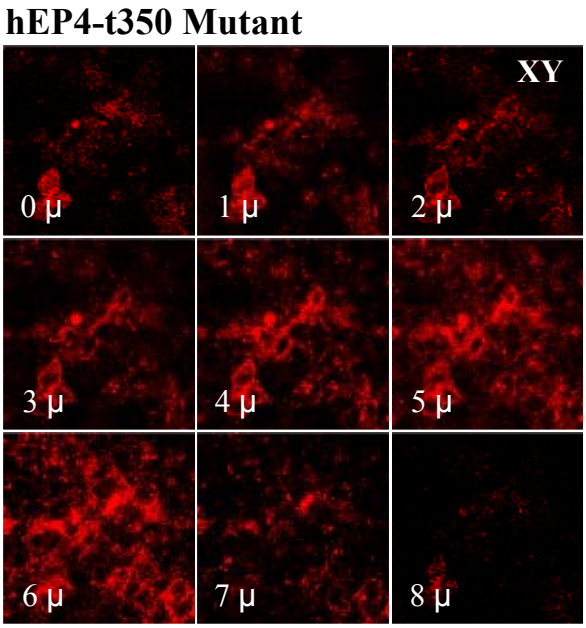

Figure 29. Confocal Microscopy Images of human bronchial epithelial (BEAS-2B) Cells Expressing the Hemagglutinin (HA)-tagged Human EP4-t350 Mutant Receptor

The panels indicate sequential confocal scans of 1 micron parallel to the monolayer (XY plane). Red fluorescence represents HA-tagged receptors. Staining of the human EP4-t350 receptor was observed at the basolateral surface of BEAS-2B cells, with lower expression observed in the intracellular space.

CHAPTER 6

DISCUSSION

Overview

The superfamily of G protein-coupled receptors (GPCRs) constitutes the largest and most diverse class of membrane bound receptors. These receptors transduce a wide range of extracellular signals, leading to numerous physiological functions throughout the body. Extensive research has been done to characterize the regulation of these receptors, focusing on aspects such as receptor desensitization, upregulation and internalization. An additional area of molecular characterization of GPCRs has been the establishment of their subcellular localization patterns in polarized epithelial cells, which is the focus of this thesis.

Several studies have investigated membrane targeting of G protein-coupled receptors using epithelial cells such as Madin-Darby canine kidney (MDCK) cells. Polarized epithelial cells are composed of apical and basolateral plasma membrane domains with specific protein compositions separated by tight junctions (Rodriguez-Boulan et al., 1989). Adrenergic (Saunders et al., 1995), muscarinic (Nadler et al., 1999), and purinergic (Wolff et al., 2004) receptors are a few examples of GPCRs that localize to specific membranes in MDCK cells; however, the underlying mechanisms are poorly understood. The membrane-specific localization of receptors is thought to play an important role in determining their physiological functions.

Previous studies in our laboratory focused on the internalization and trafficking of the human prostaglandin E_2 receptors. The four EP receptor subtypes (EP1-EP4) are all GPCRs, which differ in their second messenger pathways. The EP3 receptor is

unique in that it has eight different isoforms that differ in the lengths of the carboxyl tail. We have determined that the human EP3 isoforms, as well as the human EP2 and EP4 receptors, display different agonist-induced internalization patterns in HEK293 cells (Desai et al., 2000; Bilson et al., 2004). Additional work revealed that the EP3 isoforms internalize via different mechanisms. Specifically, EP3.I and EP3.f internalize in a clathrin-dependent manner, while EP3.II internalization is clathrin-independent (Mitchell, 2006). Moreover, we have also shown the EP3 isoforms have tissue-specific distribution patterns (Bilson, 2004).

To further extend the knowledge of the prostaglandin E_2 receptors, we examined their subcellular localization patterns in the MDCK polarized cell model. We were particularly interested in bridging the gap between receptor localization and receptor function, as PGE_2 exerts numerous physiological effects throughout the body, specifically in the kidney. In this work, we sought to determine whether differences existed in the membrane targeting profiles of the EP2 and EP4 receptors, as well as the EP3 isoforms. We also sought to identify any structural motifs within the receptor sequence that may be involved in the sorting of each receptor to its specific membrane domain. We utilized amino acid substitution, truncation and receptor chimeras in an attempt identify regions of importance within the sequences of the EP3.I, EP3.VI and EP4 receptors. Finally, we confirmed, as Wolff et al. (2004) reported, that receptor targeting is not dependent on cell type. **Table 10** summarizes our findings.

Table 10. Summary of Results

Receptor	Localization in MDCK Cells	Localization in BEAS-2B Cells
EP3.I	Apical = Basolateral = Intracellular	Apical = Intracellular
EP3.I-5A	Apical = Basolateral	Basolateral >> Apical
EP3.II	Apical = Basolateral	----------
EP3.VI	Basolateral	----------
EP3.f	Basolateral >> Apical	----------
EP2	Basolateral	Basolateral >> Apical
EP4	Basolateral	Basolateral
EP4-10ST	Basolateral > Intracellular	Basolateral
EP4-t350	Basolateral	Basolateral >> Apical
$P2Y_2$	Apical	----------
$P2Y_4$	Apical	----------
EP3.VI-$P2Y_4$	Throughout Cell	----------
$P2Y_4$-EP3.VI	Throughout Cell	----------
EP3.VI-$P2Y_2$	Apical	----------
$P2Y_2$-EP3.VI	Basolateral	----------

The aim of this work was to define the subcellular localization of the human prostaglandin E_2 receptors in polarized epithelial cells. While not within the scope of this dissertation, we believe that defining the role of receptor localization in receptor function may lead to a more complete understanding of the physiological roles of these receptors.

This discussion is arranged to illustrate the following aspects of the subcellular distribution patterns of the human prostaglandin E_2 receptors:

1. Subcellular Localization Studies of Human EP3 Isoforms.
2. Prostaglandin/Purinergic Receptor Chimeras
3. Subcellular Localization Studies of Human EP2 and EP4 Receptors.

Subcellular Localization Studies of Human EP3 Isoforms

Previous work in our laboratory, which determined the agonist-induced internalization patterns of the EP3 isoforms, also revealed that the isoforms were located on different areas of the cell surface (Bilson, 2004). Under basal conditions, the EP3.I receptor was localized to the cell surface. The other isoforms were also primarily located on the cell surface; however, isoforms III, IV and f were located just below the cell surface. Interestingly, isoform V was more centrally located within the cellular compartment. While fluorescence microscopy is not a practical option for determining localization of receptors in polarized cells, it did allow us to recognize that the isoforms do not localize exactly the same under basal conditions. To expand on this observation, we expressed the human prostaglandin EP3 isoforms in MDCK cells and examined their basal level localization patterns using confocal immunofluorescence microscopy.

Polarized MDCK cells, which are used as a model for the renal distal tubule, are commonly exploited for protein sorting studies (Herzlinger et al., 1982; Tan et al., 2004). Utilizing this cell line is further supported by the expression of prostaglandin E_2 and the EP3 receptor subtype in tubule cells. Prostaglandin E_2 is highly synthesized within the renal tubule (Bonvalet et al., 1987). Immunohistochemical studies have revealed mRNA for the EP3 receptor was expressed in the distal convoluted tubules (Morath et al., 1999), while *in situ* hybridization studies identified EP3 mRNA was densely located in the tubules in the outer medulla as well as in the distal tubules within the cortex (Sugimoto et al., 1994). Moreover, one of the prominent actions of the EP3 receptor is the inhibition of salt and water reabsorption in the collecting tubules of the kidney (Grantham and Orloff, 1968).

While the subcellular distribution of many G protein-coupled receptors has been examined using MDCK cells, studies have also been done using the EP3 isoforms of different species. In fact, Hasegawa et al. (2000) showed that the mouse EP3 isoforms display different localization patterns in MDCK cells. It was determined the mouse EP3γ receptor, which is homologous to the human EP3.II receptor isoform, is preferentially targeted to the lateral plasma membrane. The mouse EP3α and EP3β isoforms were localized in the intracellular compartment. It is also interesting to note that the composition of the C-tail of EP3γ appears to determine its targeting (Hasegawa et al., 2000).

In this work, we have shown that four human EP3 isoforms, EP3.I, EP3.II, EP3.VI and EP3.f, display unique patterns of subcellular localization in MDCK cells. The EP3.I receptor was localized throughout the cell under basal conditions, while the

EP3.II receptor localized heavily at the apical surface, with significant localization along the lateral regions of the cell. There was also a low level of expression at the basal membrane and the intracellular compartment. These two isoforms localized in a very similar pattern and it is interesting to note that the C-tail lengths of these two isoforms are also similar, as the EP3.I C-tail is 31 amino acids and the EP3.II C-tail is 29 amino acids. Both isoforms additionally share a significant number of serines and threonines within their C-tail sequence. However, when we expressed a mutant form of the EP3.I receptor containing a five amino acid substitution of SSSTS to AAAAA, there was no difference in the observed localization pattern of this mutant compared to the EP3.I wild type. This suggested that serine and threonine residues may not play a critical role in receptor localization under basal conditions. Further mutational analysis of all serine and threonine residues in both of the EP3.I and EP3.II isoforms would be necessary to further establish that these residues are not involved in receptor localization.

In contrast to the EP3.I and EP3.II isoforms, the EP3.VI isoform was expressed exclusively to the basolateral membrane. The EP3.f isoform was expressed heavily at the basolateral membrane of MDCK cells, with a low level of expression at the apical surface above the tight junctions. As these two isoforms are both heavily expressed at the basolateral surface, it was observed that both isoforms contain the same nine amino acid sequence at the end of their C-tail: **REPCSVQLS.** Truncation of this sequence would determine any role it may play in determining the localization of these isoforms.

It important to note that a GenBank search revealed homology between the C-tail of the human EP3.I isoform and the human luteinizing hormone (LH) receptor. The

amino acids highlighted in red represent identical residues, with serine and threonine residues highlighted in blue:

EP3.1 Isoform C-tail: TSLPCQCSSTLMWSDHLER*

LH Receptor C-tail: STLHCQGTALLDKTRYTEC*

The LH receptor localizes directly to the basolateral surface of MDCK cells (Beau et al., 1997), while our investigation of the EP3.I isoform revealed non-specific distribution in MDCK cells. Despite this difference, this sequence homology, although weak, may be of some relevance since sorting signals are poorly defined. Mutational analysis to confirm the significance of these residues was not attempted as further investigation revealed that Beau et al. (2004) identified the basolateral sorting signal for the LH receptor at the distal part of the intracellular domain.

Additionally, we expressed these four EP3 isoforms and the EP3.I mutant receptor in the human lung epithelial cell line, BEAS-2B, and observed near identical localization patterns. This data was in accordance with other studies (Wolff et al., 2004) that have shown receptor localization is not dependent on cell type.

The human EP3 isoforms provide a natural model to examine the role of the C-tail region in receptor localization studies. These eight isoforms differ only in C-tail length and composition, ranging from 6 to 66 amino acids in length (Kotani et al., 1997). We believe that because the isoforms share complete sequence homology across the first 359 amino acids, their C-tails must contain a sorting signal(s) required to traffic these isoforms to their proper membrane domain(s). The information we have obtained regarding the differential localization of the human EP3 isoforms may explain why a heterogeneous population of receptor isoforms exists and supports our hypothesis that

membrane domain-specific expression of these isoforms is related to their physiological functions.

Prostaglandin/Purinergic Receptor Chimeras

To further characterize the role of the human EP3 isoform C-tail in receptor localization, we constructed receptor chimeras between the basolaterally targeted EP3.VI isoform and two apically targeted purinergic receptor subtypes, $P2Y_2$ and $P2Y_4$. Like the prostaglandin E_2 receptors, purinergic receptors are also seven transmembrane G protein-coupled receptors. Using chimeras between similar proteins with opposite phenotypes is more useful than deletion or truncation mutagenesis because it reduces the possibility that a change in receptor targeting is caused by a generalized effect on the protein structure (Nadler et al., 2001).

Purinergic receptors are a family of plasma membrane molecules activated by adenosine and extracellular nucleotides, such as ATP. There are three classes of purinergic receptors: P1, P2Y and P2X. P2X receptors are ligand-gated ion channels, while the P1 and P2Y receptors are G protein-coupled metabotropic receptors (Harden, 1998; Ralevic and Burnstock, 1998). The G protein-coupled receptors are further distinguished by their endogenous agonist sensitivity. P2Y receptors are preferentially stimulated by ATP, whereas P1 receptors are preferentially activated by adenosine (Bloom, 1996).

Molecular studies have determined that the human P2Y receptor exists as eight subtypes: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ (Lee and O'Grady, 2003). These subtypes are further divided into two subfamilies based on their second

messenger pathways. The $P2Y_1$ family is comprised of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors, which couple to G_q, activating phospholipase C (Ralevic and Burnstock, 1998). The $P2Y_{12}$ subfamily includes $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$, which activate $G_{i/o}$ and cause an inhibition of adenylyl cyclase (Communi et al., 2001).

We first attempted a gain-of-function approach to study the EP3.VI and $P2Y_4$ receptors. The $P2Y_4$ receptor achieves apical targeting through a sorting signal recognized by the cell that is located in its C-terminal tail (Qi et al., 2005). We anticipated that each chimera would be directed to the membrane domain associated with its C-tail: EP3.VI-$P2Y_4$ would localize to the apical surface, while $P2Y_4$-EP3.VI would localize to the basolateral surface. Unfortunately, we observed unsorted distribution in MDCK cells for both of these chimeras, with localization observed throughout the cells. While this observation was surprising at first, there are quite a few possible reasons for this result. Many studies suggest that apical sorting signals consist of protein sequences in the extracellular, transmembrane, and/or cytoplasmic domains (Chuang et al., 1998; Jacob et al., 1999; Kundu et al., 1996; Muth et al., 1998; Tugizov et al., 1998). On the contrary, basolateral sorting signals are more commonly found in the C-tail region (Matter et al., 1994). While Qi et al. (2005) did show that the apical signal for the $P2Y_4$ receptor was located in its C-tail by truncating the $P2Y_4$ receptor, it is quite possible that the creation of the EP3.VI-$P2Y_4$ chimera disrupted apical targeting due to unknown signals located within the transmembrane or loop regions of the EP3.VI receptor. Additionally, the targeting of these chimeras may in part be affected by the G protein to which each receptor couples. While the EP3.VI isoform couples preferentially to the $G_{i/o}$ pathway, the $P2Y_4$ receptor couples to the G_q pathway. Qi et al. (2005) utilized receptor

chimeras between the $P2Y_4$ receptor and the B2 bradykinin receptor, which also couples to the G_q pathway in most cells (Austin et al., 1997; Ricupero et al., 1997; Yang et al., 1999). Furthermore, Saunders and colleagues (1998) generated chimeras between the alpha-2A adrenergic receptor and the A1 adenosine receptor, both of which couple to the inhibitory G_i pathway.

Although the second messenger pathway does not predict patterns of subcellular localization, it is not clear what role, if any, the G protein plays in receptor localization. Nadler and colleagues (2001) determined that the basolateral sorting signal of the M_3 receptor was located in the third intracellular loop, while the third intracellular loop has also been shown to play an important role in functional coupling to the G_q protein (Blin et al., 1995 and Kostenis et al., 1997). Chimeras of M_2 and M_3 muscarinic receptors, which localize to opposite surfaces and couple to G_i and G_q respectively, revealed that the basolateral sorting signal of the M_3 receptor was not enough to cause the M_2 receptor to couple to the G_q pathway (Nadler et al., 2001). More studies must be conducted in order to clarify the relationship between G protein signaling and receptor localization.

Additionally, we constructed chimeras between the EP3.VI isoform and the $P2Y_2$ purinergic receptor, which contains an apical sorting signal in its first extracellular loop (Qi et al., 2005). As we strongly believe a basolateral sorting signal is located in the C-tail of the EP3.VI isoform, the EP3.VI-$P2Y_2$ chimera likely contained no known sorting signals. In contrast, the $P2Y_2$-EP3.VI chimera potentially contained two opposing sorting signals (apical and basolateral) within its sequence. This study revealed that the EP3.VI-$P2Y_2$ chimera localized exclusively to the apical surface of MDCK cells. This finding suggests, in our system, receptors containing no known sorting signals will be targeted to

the apical surface by default. On the other hand, the $P2Y_2$-EP3.VI receptor chimera, which presumably contains an apical sorting signal in the first extracellular loop and a basolateral sorting signal in the C-tail, localized rather robustly to the basolateral surface. This observation is in agreement with several other studies that have shown basolateral sorting signals can be dominant over apical signals in the same molecule (Hobert et al., 1997; Le Bivic et al., 1991; Matter et al., 1992).

The receptor chimera studies provided valuable insight on the role of the C-tail of the EP3.VI receptor. While we have not succeeded in determining specific amino acid motifs or residues critical for the proper sorting of the EP receptors, we believe there is significant evidence that the EP3.VI C-tail is involved in the trafficking of this isoform to the basolateral surface of polarized cells under basal conditions.

Subcellular Localization Studies of Human EP2 and EP4 Receptors

Upon agonist stimulation, the human EP2 and EP4 receptors both couple to the G_s pathway to stimulate adenylyl cyclase and increase cAMP (Bastien et al., 1994; Regan et al., 1994). While both of these receptors have a similar affinity for PGE_2, these receptors have distinct characteristics pertaining to their structure and regulation. Structurally, the 358-amino acid EP2 receptor has a short third intracellular loop and C-tail compared to the 488-amino acid EP4 receptor. Additionally, the EP4 receptor undergoes both short-term agonist-induced desensitization and internalization, while EP2 does not (Nishigaki et al., 1996; Bastepe and Ashby, 1997; Desai et al., 2000; Desai and Ashby; 2001;). Our laboratory also determined that the C-tail of the EP4 receptor was involved in receptor

internalization but serine and threonine residues did not play a role in this process (Desai et al., 2000).

To further compare the EP2 and EP4 receptors, we created retroviral constructs containing these receptors and observed their subcellular expression in MDCK cells. We observed both receptors localized to the basolateral domain during steady-state. In an attempt to locate sorting signals, we focused our efforts on the EP4 receptor as we had two receptor mutants that were previously prepared by Bastepe and Ashby (1999): the hEP4-10ST mutant and the hEP4-t350 mutant. When expressed in MDCK cells, both of these mutants localized predominantly to the basolateral surface, again implying that serine and threonine residues do not play a role in receptor localization. It was interesting to find that the C-tail truncation mutant, hEP-t350, did not have an altered localization pattern because, as mentioned previously, many basolateral sorting signals are located within the cytoplasmic tail of transmembrane receptors (Matter et al., 1994). However, Nadler (2001) discovered a novel basolateral targeting motif for the M_3 muscarinic receptor located in the third intracellular loop. Moreover, Saunders et al. (1998) determined that there are multiple, noncontiguous membrane-embedded sequences of the alpha-2A adrenergic receptor located within or near the lipid bilayer that target this receptor to the basolateral surface. Our examination of the human EP4 receptor concludes that the basolateral sorting signal for the EP4 receptor is located within the first 350 amino acids of its sequence and not within its C-tail. As serine and threonine residues were not involved in the internalization of EP4, they also do not play a role in receptor localization during steady-state. Interestingly, they were involved in the

internalization of the EP3.I isoform but, again, did not effect the localization of the receptor.

As we did for the EP3 wild type and EP3.I-5A mutant receptors, we expressed the EP2 wild type, EP4 wild type, hEP4-10ST and hEP4-t350 mutant receptors in the human lung epithelial cell line, BEAS-2B, and observed similar localization patterns as the MDCK cells. By comparing the localization of three wild type and three mutant receptors in the BEAS-2B cell line to the MDCK cell line, we strongly believe receptor localization is not a function of cell type. While our findings are in agreement with Wolff et al. (2004), expression of these receptors in a third cell line may further substantiate our observations.

Summary and Conclusion

This thesis examined the differences in subcellular localization of the human prostaglandin E_2 receptors in polarized epithelial cells. Using confocal immunofluorescence microscopy, we have shown that four EP3 isoforms, as well as the EP2 and EP4 receptors, have distinct and unique subcellular localization patterns in Madin-Darby canine kidney (MDCK) cells. Additionally, we observed the subcellular localization patterns of three mutant receptors and determined that serine and threonine residues within the EP3.I and EP4 C-tails do not play a critical role in targeting these receptors to their respective membrane domains. We also concluded that the basolateral sorting signal for the EP4 receptor is not located within the last 138 amino acids of the sequence, as truncation of the C-tail at amino acid 350 did not alter the basolateral targeting of the receptor. The localization patterns for the wild type

receptors and mutants in human lung epithelial cells (BEAS-2B) were quite similar to the MDCK cells, suggesting that receptor localization is not dependent on cell type. Finally, in an attempt to locate structural motifs responsible for apical or basolateral localization, receptor chimeras between the purinergic $P2Y_2$/$P2Y_4$ receptors and EP3.VI isoform were constructed. Overall, these studies show that the human prostaglandin E_2 receptors display unique subcellular localization patterns and may contain distinct sorting signals within their amino acid sequence.

Future Studies

We believe the work presented in this dissertation contributes new and useful information regarding the molecular characterization of the human prostaglandin E_2 receptors and provides a solid foundation for future studies.

Utilizing confocal immunofluorescence microscopy, we have shown that the human PGE_2 receptors display unique localization patterns in polarized epithelial cells. To quantitatively measure receptor localization, biotinylation studies would be useful to determine the levels of receptor expression at the apical or basolateral surface.

We have made an extensive effort to examine molecular components within the receptor sequence to determine the mechanism by which these receptors are targeted to specific membrane domains in epithelial cells; however, this remains an essential question in molecular and epithelial cell biology. By creating receptor chimeras between the purinergic $P2Y_2$/$P2Y_4$ receptors and the human EP3.VI isoform, we observed rather interesting and unexpected localization patterns in MDCK cells. A broad range of chimera studies could be utilized to further elucidate specific targeting signals located

within the receptor sequence. To account for any differences in subcellular localization that may be caused by the specific G protein to which the receptor couples, it may be important to create chimeras between receptors based on their second messenger pathways. For example, the muscarinic M_2 and M_4 receptors, which couple to $G_{i/o}$, may provide more valuable information regarding the targeting of the EP3 isoforms, which also couple to $G_{i/o}$.

A potential caveat to using receptor chimeras or mutated receptors is the possibility of creating non-functional receptors. Measuring adenylyl cyclase activity or intracellular calcium levels would determine functional activity of these receptors.

While our efforts focused on the steady-state localization of the human PGE_2 receptors, additional work may focus on the trafficking patterns of these receptors following agonist stimulation. We have previously shown that the PGE_2 receptors display unique agonist-induced internalization patterns in HEK293 cells. Utilizing the MDCK cell model, we could determine if this phenomenon is consistent in polarized epithelial cells.

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