IDENTIFICATION OF TARGETING SIGNALS IN HUMAN P2Y RECEPTORS IN POLARIZED MDCK(II) EPITHELIAL CELLS

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ABSTRACT

SAMUEL CAREY WOLFF: Identification of Targeting Signals in Human P2Y Receptors in Polarized MDCK(II) Epithelial Cells
(Under the direction of Robert A. Nicholas, Ph.D.)

P2Y receptors, which belong to the G-protein coupled receptor superfamily, play prominent roles in epithelial cell physiology, such as regulated ion transport and response to stress. Published studies utilizing indirect, pharmacology assays suggested a polarized distribution of P2Y receptors in a variety of epithelial cells. Therefore, we examined directly the distribution pattern of the entire P2Y receptor family in MDCK(II) epithelial cells by confocal microscopy as well as the localization of the Gq-coupled P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$) in epithelial cells from lung and colon. Our results showed that seven of the eight receptor subtypes are localized to either the apical or basolateral membrane surface of MDCK(II) cells. Moreover, a nearly identical pattern of distribution was observed in the other epithelial cell types (Wolff et al., 2005). The polarized targeting of cell-surface proteins is mediated by the protein-sorting machinery of the cell, which reads and interprets targeting signals contained within the primary sequence of polarized proteins and ensures delivery to the correct subcellular location. We postulated that P2Y receptors contain targeting signals that direct their polarized sorting in epithelial cells. To test this hypothesis, we analyzed a series of P2Y receptor mutants and chimeras, which allowed us to locate the targeting signals for all of the polarized P2Y receptor subtypes. Once the locations of the apical or basolateral targeting signals were determined, we fully characterized the

basolateral targeting signal of the $P2Y_1$ receptor and the apical targeting signal of the $P2Y_4$ receptor, both of which are located in the C-terminal tail. The results of these studies demonstrated that the basolateral signal of the $P2Y_1$ receptor is 25 amino acids in length and functions in a sequence-independent manner, with charged residues playing a key role in targeting, while the apical signal of the $P2Y_4$ receptor is 23 amino acids long with no remarkable features or key amino acids identified as of yet. In this dissertation, we describe a series of experiments that completely characterized the apical and basolateral signals of these two purinergic receptors.

To my family whose love and support sustained me throughout the years and motivated me to complete this incredible journey. I dedicate this work to my parents, Peter and JoAnn Wolff, to Ethel Fishman and Melba Levinsky who left us much too soon and would have been proud to see this work complete and to my wife and son, Amy and Emmett, whose love help me through the most difficult times and for this they share in this great achievement.

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LIST OF ABBREVIATIONS

2MeSADP	2-methylthio-ADP
AC	adenylate cyclase
ADP	adenosine 5’-diphosphate
AP	adaptor protein
APC	apical targeting cassette
aPKC	atypical protein kinase C
ATP	adenosine 5’-triphosphate
BK_2	bradykinin 2 Receptor
BLC	basolateral targeting cassette
cAMP	cyclic adenosine 3’,5’- monophosphate
CD	circular dichroism
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
C-tail	C-terminus tail
DIG	detergent-insoluble glycosphingolipid-enriched membrane domain
DMEM	Dulbecco's Modified Eagle's Medium
DRM	detergent-resistant membrane
EAAT3	excitatory amino acid transporter 3
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
E-NPP	ecto-nucleotide pyrophosphatase/phosphodiesterase
E-NTPDase	ecto-nucleoside 5’-triphosphate diphosphohydrolase

FBS	fetal bovine serum
fmol	femto mole
FSHR	follicle stimulating hormone receptor
GCC	guanylyl cyclase C
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
GPI	glycosylphophatidylinositol
GRK	G protein-coupled receptor kinase
HA	hemagglutinin epitope tag sequence YPYDVPDYA
HRP	horseradish peroxidase
IP3	inositol triphosphate
I_{sc}	short circuit current
mAb	monoclonal antibody
MDCK(II)	Madin-Darby canine kidney type II cells
mg	milligram
MRS2179	2'-Deoxy-N^6-methyladenosine-3',5'-bisphosphate
MRS2279	2'-chloro-N^6-methyl-(N)-methanocarba-2'-deoxyadenosine 3',5'-bisphosphate
NaCl	sodium chloride
N-CAM	neural cell adhesion molecule
NHERF	Na exchanger regulatory factor
NMR	nuclear magnetic resonance
NSF	N-ethylmaleimide-sensitive factor

N-term	N-terminus
PAR	partitioning-defective protein
PBS	phosphate buffered saline
PDZ	PSD-95/Drosophilia discs/ZO-1
PFA	paraformaldehyde
pIgR	polymeric immunoglobin receptor
PIP_2	phosphotidyl inositol-3,4-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLAP	placental alkaline phosphatase
PLC	phospholipase C
PM	plasma membrane
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNAP	soluble N-ethylmaleimide-sensitive factor
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
Sulfo-NHS-SS-Biotin	sulfosuccinimidyl 2-(biotinamido)-ethyl-1, 3-dithiopropionate
TSHR	thyrotropin stimulating hormone receptor
UDP	uridine 5'-diphosphate
UDP-Glucose	uridine 5'-diphosphate glucose
UTP	uridine 5'-triphosphate

CHAPTER I: General Introduction

1. P2 Receptor Background

The effects of extracellular nucleotides were first described nearly 80 years ago when Drury and Szent-Gyorgyi demonstrated that extracellularly applied adenine nucleotides induced coronary vasodilation (Drury and Szent-Györgyi, 1929). Several decades later a second physiological role for extracellular nucleotides was demonstrated by showing that ADP promoted platelet aggregation (Born, 1962; Gaarder et al., 1961). The concept of nucleotides as signaling molecules activating their cognate receptor(s) was not established and accepted until many years later. In fact, this rich field now known as 'purines' research would probably not have been established, at least in its current form, had it not been for the persistent work of Geoff Burnstock throughout the 1970s and 1980s (Burnstock, 1972; Burnstock and Kennedy, 1985). Because of the work of Burnstock and many others, we now know that extracellular nucleotides promote a myriad of physiological responses in most cells and tissues, including ion transport, platelet aggregation, contraction of smooth muscle and depolarization of neurons (Harden et al., 1995; Ralevic and Burnstock, 1998).

By the mid-1990s numerous published reports demonstrated that extracellular nucleotides activated membrane-bound P2 receptors with two distinct signal transduction mechanisms, leading to the proposal that two separate receptor families, named P2X and P2Y, be established to reflect these differences (Abbracchio and Burnstock, 1994). Subsequent cloning of both types of P2 receptors confirmed the existence of these two

protein families, with P2X receptors as fast-acting ionotropic cation channels and the slower-acting metabotropic P2Y receptors that belong to the G-protein coupled receptor (GPCR) superfamily. To date, seven P2X receptors ($P2X_{1-7}$) have been characterized, while eight P2Y receptors ($P2Y_{1,2,4,6,11,12,13 \text{ and } 14}$) have been cloned, with a closely related orphan receptor (GPR87) thought to be the ninth and most likely final member.

1.1 Cloned P2X Receptors

As described above, P2X receptors are fast-acting ionotropic cation channels with seven members ($P2X_{1-7}$) cloned to date. Moreover, there are no reports of additional homologous P2X sequences, strongly suggesting that seven is the total number of members for this receptor family. These receptors are multimeric structures that are assembled with the same (homo-oligomers) or different (hetero-oligomers) subunits, with the most convincing evidence suggesting that the subunits form trimers (Khakh and North, 2006; Nicke et al., 1998). The proposed membrane topology of these receptors is one with a large extracellular region flanked by two transmembrane domains and with both the N- and C-terminus within the cytoplasmic space (Brake et al., 1994; Valera et al., 1994). Unlike P2Y receptors, P2X receptors are activated exclusively by ATP (as well as numerous ATP analogues) and upon activation mediate the influx of divalent ions such as Ca^{2+} and Mg^{2+}. P2X receptors are widely expressed and include tissues such as smooth and skeletal muscle, leukocytes, lymphocytes, as well as the central and peripheral nervous systems. Given the nearly ubiquitous expression of these receptors throughout the body, P2X receptors have been implicated in a variety of physiological functions including contraction of smooth muscle and

the bladder, modulation of pain sensation/nociception (Fukui et al., 2006; Krishtal et al., 2006) and regulation of inflammation (Lister et al., 2007).

1.2 G protein-coupled receptors (GPCRs)

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are signal transducers that connect cell-surface receptors to effectors and their intracellular signaling pathways. The three subunits that comprise a functional G-protein are α, β and γ, with the α subunit containing the guanine nucleotide-binding site. Receptors that couple to these signal transducers are known as G protein-coupled receptors (GPCRs) and they constitute the largest and most diverse type of membrane-bound proteins. Genes encoding members of the GPCR superfamily, with their trademark seven-transmembrane helical domains, represents approximately 5% of the mammalian genome, making it one of the largest gene families. These receptors are activated by an extremely diverse set of ligands/stimuli (photons, hormones, neurotransmitters, nucleotides, Ca^{2+}, amino acids, peptides, proteins and chemokines) but couple to a limited number of heterotrimeric G proteins (Gαβγ). There are 20 Gα subunits that are grouped into four families: Gαs, Gαi/o, Gαq/11 and Gα12/13. In contrast, 5 Gβ and 11 Gγ subunits form an obligate βγ dimer (Neves et al., 2002).

Upon activation by their cognate ligands, GPCRs undergo a conformational change that promotes the exchange of GDP for GTP at the Gα subunit, which moves the G protein from the inactive to the active state. Once activated, Gα subunits dissociate away from both the receptor and Gβγ complex to initiate a variety of signaling pathways that regulate numerous physiological processes, including chemotaxis, gene transcription, enzymatic

activity and activation of ion channels and transporters. Moreover, the switching of GPCRs between the activated (on) and deactivated (off) state is exquisitely modulated by a host of proteins (e.g. GRK, spinophilin, calmodulin and arrestins) making this receptor system one of the most tightly regulated biological systems (Bockaert et al., 2004).

1.21 G proteins

The cyclic AMP signaling pathway was the first cell signaling pathway described many decades ago (Sutherland and Wosilait, 1955), which we now know is mediated by the Gαs protein. Activation of this G protein leads to the activation of adenylate cyclase (AC) and the production of the second messenger 3',5'-adenosine monophosphate (cAMP), which stimulates other down-stream effectors including protein kinase A (PKA). In contrast to the stimulatory nature of the Gs pathway, the Gi/o pathway serves to inhibit adenylate cyclase activity and, therefore, suppress the production of cAMP second messenger. In addition, Gβγ subunits released from Gi/o can also signal through activation of PLC-β isozymes (particularly PLC-β3 and –β2) and G protein-regulated inwardly rectifying K^+ (GIRK) channels. Activation of Gαq leads to stimulation of phospholipase C (PLC) activity that hydrolyzes phosphotidyl inositol-3,4-bisphosphate (PIP_2) into two second messengers, diacylglycerol and IP_3, which subsequently activate protein kinase C isozymes and promote mobilization of intracellular calcium. The signaling pathways for Gα12 and Gα13 is less clear considering most experiments have taken place transfected cell lines (Neves et al., 2002). In spite of these experimental limitations, it is generally thought that in native systems Gα12 stimulates phospholipase D, c-Src and PKC (Jiang et al., 1998), while Gα13 has been shown to directly activate the guanine nucleotide exchange factor for the GTPase Rho, thus

leading to a variety of effects including regulation of the Na^+-H^+ exchanger (Shi and Kehrl, 2001). Taken together, these distinct G proteins and their unique signaling pathways serve to modulate many physiological processes as described above.

1.3 Cloned P2Y Receptors

The cloning and characterization of P2Y receptors began in earnest in the early 1990s with the advent of molecular biology. Once the first P2Y receptor subtype ($P2Y_1$) was identified, subsequent searches and cloning of additional members was facilitated by sequence homology searches and northern blot analyses. Gaps in the P2Y receptor nomenclature are the consequence of misidentifying orphan receptors as P2Y receptors and having to withdraw the designations later. The molecular identification, pharmacological profiles and physiological consequences following receptor activation of the eight P2Y receptor subtypes are described below.

Eight functional G protein-coupled P2Y receptor subtypes ($P2Y_{1,2,4,6,11,12,13,14}$) exist and these receptors fall into two sub-families on the basis of their signaling properties and sequence identities. The $P2Y_1$ receptor family, comprising of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors, activates heterotrimeric G proteins of the G_q family, thereby activating phospholipase C, causing generation of inositol phosphates and mobilization of intracellular Ca^{2+} stores (Harden, 1998; Ralevic and Burnstock, 1998). In addition to coupling to activation of phospholipase C, the $hP2Y_{11}$ receptor is also coupled to Gs and activation of adenylyl cyclase (Communi et al., 1997; Qi et al., 2001a; Torres et al., 2002). In contrast, the $P2Y_{12}$ receptor family, comprising $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors, activates Gi/o,

thereby promoting the inhibition of adenylyl cyclase activity (Fig. 1) (Chambers et al., 2000; Communi et al., 2001; Hollopeter et al., 2001; Zhang et al., 2002).

1.3A $P2Y_1$ receptor

The first P2Y receptor identified was the $P2Y_1$ receptor, which was originally cloned from chicken (Webb et al., 1993), and subsequently homologues were identified in human (Ayyanathan et al., 1996; Schachter et al., 1996), rodent (Tokuyama et al., 1995), cow (Henderson et al., 1995), turkey (Filtz et al., 1994) and skate (Dranoff et al., 2000). Tissue distribution for this receptor is wide and includes platelets, brain tissue, skeletal muscle and the digestive tract. The $P2Y_1$ receptor is activated potently by adenine dinucleotides (most notably 2MeSADP) and to a much lesser extent by adenine trinucleotides, leading to activation of the Gq signaling pathway (described above). One of the primary physiological roles for the $P2Y_1$ receptor is aggregation of platelets. This was demonstrated by the distinct phenotype found in the $P2Y_1$ knock-out mouse, which displayed decreased platelet aggregation, increased bleeding time and resistance to thromboembolism (Fabre et al., 1999; Leon et al., 1999). Other physiological roles for this receptor includes the regulation of voltage-gated ion channels (Lee et al., 2003b), vasodilation via stimulation of nitric oxide (Guns et al., 2005) and potentially the modulation of pain pathways (Yajima et al., 2005).

1.3B $P2Y_2$ receptor

The $P2Y_2$ receptor was the second P2Y receptor cloned, but was the first uracil nucleotide-activated member of this receptor family identified. The $P2Y_2$ receptor was

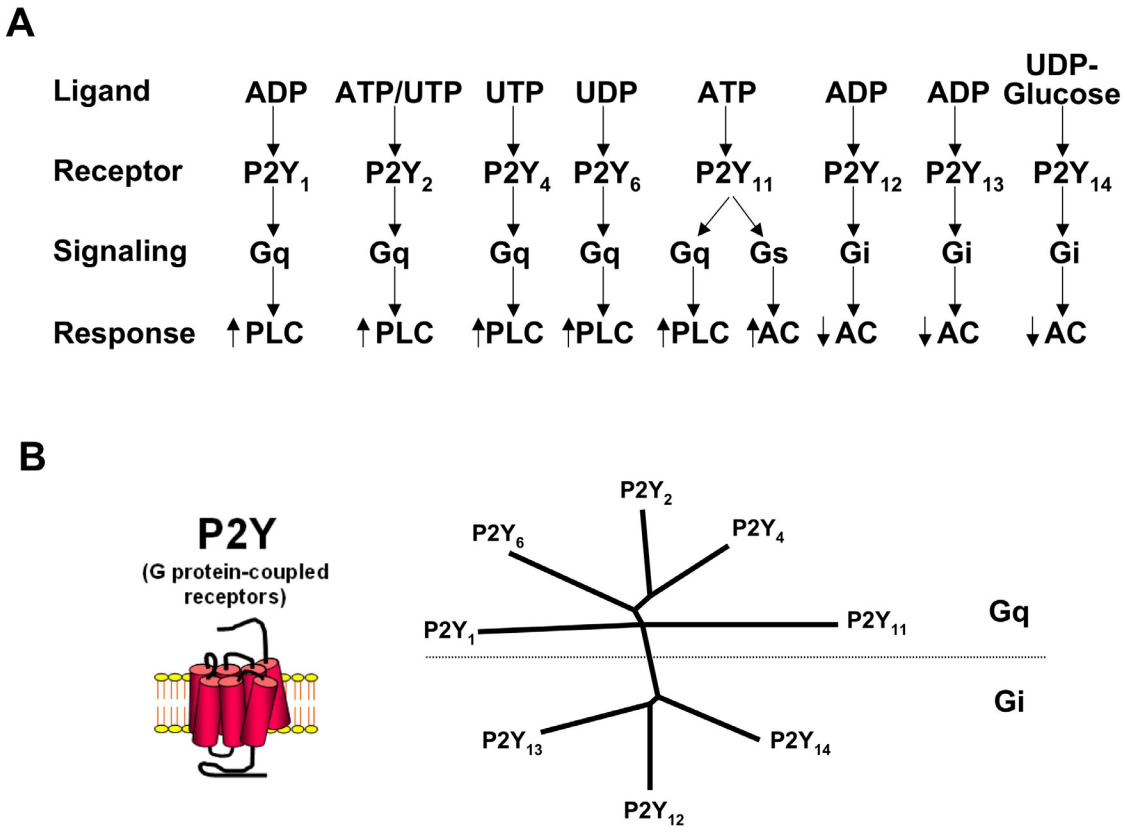


Figure 1. Cloned P2Y receptor family agonists, signaling and topology.

A) The agonist profile for all the human P2Y receptors cloned to date along with the signaling pathway(s) for each receptor subtype is shown. These properties are discussed in detail in the text. **B)** The topology of this seven transmembrane protein is shown as well as a dendrogram for the receptor family, which is divided into two sub-families: $P2Y_1$ and $P2Y_{12}$.

originally cloned from the mouse in 1993 (Lustig et al., 1993) followed by the human homologue in 1994 (Parr et al., 1994a). The $P2Y_2$ receptor has a wide tissue distribution with prominent expression in the lung, heart, skeletal muscle, spleen and kidney. Early pre-cloning functional studies suggested the existence of this class of receptors (O'Connor et al., 1991), and the name 'P2U' was coined based on the pharmacological profile as P2 receptors that are potently activated by uracil nucleotides, e.g. UTP (this name was kept until the formal P2Y nomenclature was introduced). In addition to potent activation by UTP, this receptor is equally activated by ATP lending it a unique pharmacological profile that is often utilized as a signature for identification (McAlroy et al., 2000; Nicholas et al., 1996).

Even before this receptor was cloned and formally recognized, published experiments by Boucher and colleagues demonstrated a physiological role for this receptor as a regulator of Cl^- secretion in airway epithelia (Boucher et al., 1989; Mason et al., 1991). In addition to airway epithelia, $P2Y_2$ receptors carry out a similar physiological role in nasal epithelia (Gayle and Burnstock, 2005) as well as in cells found in the lacrimal gland and the gastrointestinal tract (Cowlen et al., 2003; Matos et al., 2005). Most importantly, it was quickly recognized that the physiological role of $P2Y_2$ receptors as a regulator of Cl^- secretion in airway epithelia could be used as a therapeutic target in the treatment of cystic fibrosis (CF) (Donaldson and Boucher, 1997; Parr et al., 1994b). It was hypothesized that activation of the $P2Y_2$ receptor and its downstream Cl^- channel could serve to restore the aqueous layer in the lumen of airway cells, a role normally carried out by the cystic fibrosis transmembrane regulator (CFTR) but which is defective in CF patients. Indeed, this idea has come to fruition as a $P2Y_2$ receptor agonist has been developed and currently is in phase III testing for the treatment of CF (Kellerman et al., 2002).

1.3C $P2Y_4$ receptor

The $P2Y_4$ receptor was identified independently by two different groups in 1995 (Communi et al., 1995; Nguyen et al., 1995) and shown to be a second Gq-coupled P2Y receptor activated by uracil nucleotides. Initially, the tissue distribution of this receptor was shown to be restricted to the placenta and pancreas. However, subsequent reports demonstrated expression of either $P2Y_4$ receptor mRNA or protein in the human lung (Communi et al., 1999), murine stomach, intestine and liver (Suarez-Huerta et al., 2001), and in the epithelium of the gerbil inner ear (Marcus and Scofield, 2001; Sage and Marcus, 2002). The $P2Y_4$ receptor has a particularly interesting pharmacological profile that is species-dependent. The human orthologue of this receptor is selectively activated by UTP and fully antagonized by ATP, whereas the rodent (rat and mouse) orthologues are activated equipotently by both ATP and UTP (Herold et al., 2004; Kennedy et al., 2000). One of the physiological roles for this receptor is regulation of epithelial ion transport (Matos et al., 2005; McAlroy et al., 2000; Robaye et al., 2003). For example, Robaye and colleagues demonstrated that the $P2Y_4$ receptor is a dominant regulator of salt and fluid transport in the jejunum of the small intestine (Robaye et al., 2003). This homeostatic regulation of ions and fluid by epithelium in the small intestine is key to the normal functioning of this tissue and serves to underscore the important role nucleotide receptors, in this case the $P2Y_4$ receptor, play in this process (Leipziger, 2003).

1.3D $P2Y_6$ receptor

A third uridine-selective receptor, the $P2Y_6$ receptor, was cloned in 1995 from rodent and in 1996 from humans (Chang et al., 1995; Communi et al., 1996). This receptor is

activated potently by UDP but weakly if at all by ADP, ATP and UTP (Nicholas et al., 1996). This receptor is widely distributed, including the heart, spleen, placenta, aorta, brain and intestine, and is thought to be involved in several important immune-related functions (Communi et al., 1996; Somers et al., 1998). More specifically, this receptor is expressed in T-cells and has been implicated as a mediator for the infiltration of these cells into the colon, which can damage the vital epithelium lining this organ and leading to inflammatory bowel disease (Somers et al., 1998). In addition, the $P2Y_6$ receptor has been shown to regulate the secretion of NaCl in colonic epithelium, which helps to maintain an ion gradient that is key to the normal functioning of this tissue (Kottgen et al., 2003). Most recently, it has been demonstrated that damage to neurons and microglia causes the upregulation of $P2Y_6$ receptors in neighboring microglia, which are thought to help regulate the critical housekeeping function of microglia phagocytosis (Koizumi et al., 2007).

1.3E $P2Y_{11}$ receptor

The human $P2Y_{11}$ receptor was first identified by Communi *et al.* in 1997 and was reported to couple to both phospholipase C and adenylyl cyclase signaling pathways (Communi et al., 1997). Additional work confirmed that this receptor coupled to both the Gq and Gs signaling pathways, but demonstrated that the receptor coupled to activation of phospholipase C at much lower levels of agonist than to activation of adenylyl cyclase (Qi et al., 2001a; Torres et al., 2002). Surprisingly, rodents do not contain sequences similar to the $P2Y_{11}$ receptor; however, in 2001 a canine $P2Y_{11}$ receptor homologue was cloned and, similar to the human receptor, was shown to couple to both phospholipase C and adenylyl cyclase. The canine orthologue of the $P2Y_{11}$ receptor is only 70% identical to its human

counterpart and displays interesting differences in its pharmacological properties. The human $P2Y_{11}$ receptor is activated more potently by adenosine triphosphates than by adenosine diphosphates and is insensitive to 2-thioether substitutions in the adenine ring, whereas the canine receptor is activated more potently by nucleoside diphosphates than by adenosine triphosphates and is highly sensitive to 2-thioether substitution (Qi et al., 2001b; Zambon et al., 2001). Evidence for the physiological role for this receptor suggests it may be involved in the modulation of dendritic cell differentiation as well as mediating the stress response in epithelial cells (Insel et al., 2001; Wilkin et al., 2001; Zambon et al., 2001).

1.3F $P2Y_{12}$ receptor

The existence of this receptor was suspected for many years before its cloning and formal designation as a P2Y receptor due to its prominent role in platelet aggregation. Starting in the early 1960s, application of ADP to platelets was shown to induce aggregation through an unidentified receptor, named the P2T receptor. In the ensuing years, it was demonstrated that ADP promoted both inhibition of adenylate cyclase as well as release of intracellular calcium in platelets, which led to the idea that there was one receptor (P2T) that coupled to two signaling pathways or that there were two receptors with similar agonist profiles that coupled separately to the different pathways. The receptor coupled to phospholipase C and intracellular Ca^{2+} mobilization was eventually identified as the $P2Y_1$ receptor (Gachet et al., 1995; Hechler et al., 1998a; Hechler et al., 1998b; Leon et al., 1999), thus supporting the idea that ADP activated two distinct receptors in platelets, but the receptor coupled to inhibition of adenylyl cyclase remained elusive.

Given its central role in platelet aggregation, the receptor coupled to adenylyl cyclase was a target for antithrombotic drugs such as clopidogrel even before its molecular identity was uncovered. Eventually, in 2001, three groups independently identified a Gi-coupled receptor that was expressed in platelets and was involved in platelet aggregation. The $P2Y_{12}$ receptor, which was also named $P2Y_{ADP}$, $P2Y_{AC}$, $P2Y_{CYC}$ and $P2T_{AC}$, together with the $P2Y_1$ receptor, was shown to be necessary for ADP-promoted platelet aggregation (Hollopeter et al., 2001; Takasaki et al., 2001; Zhang et al., 2001). The tissue expression of the $P2Y_{12}$ receptor was shown to be limited to platelets and neuronal tissue. The $P2Y_{12}$ receptor is activated by adenosine diphosphates such as 2MeSADP and antagonized by adenosine monophosphates such as 2MeSAMP. The agonist profiles for $P2Y_1$ and $P2Y_{12}$ receptors are sufficiently similar that for many years it was thought these two receptors were a single receptor; however, the two receptors are most dissimilar in their antagonist profiles with very little overlap in activity.

1.3G $P2Y_{13}$ receptor

Since its identification in 2001 as a Gi coupled-receptor activated by ADP (Communi et al., 2001; Zhang et al., 2002), this member of the human P2Y receptor family has the distinction of being the least well studied. To date, no physiological and/or therapeutic role has been assigned unequivocally to this receptor, although its prominent expression in the brain, lymph nodes, bone marrow, spleen and liver suggests a role in the immune response and/or hematopoiesis. For example, a recent study has suggested that the $P2Y_{13}$ receptor may play a role in the regulation of cholesterol homeostasis and is potentially a therapeutic target in the treatment of atherosclerosis (Jacquet et al., 2005). Finally, a mouse orthologue was

cloned as well, demonstrating the existence of this receptor in at least two different mammalian species (Fumagalli et al., 2004).

1.3H $P2Y_{14}$ receptor

The $P2Y_{14}$ receptor is the most recent addition to the P2Y receptor family. The receptor is unique in that it is activated by UDP-sugars such as UDP-glucose, UDP-galactose, and UDP-N-acetylglucosamine (Chambers et al., 2000). Originally, this receptor was not deemed a *bona fide* P2Y receptor, but given its relatively high sequence homology to the $P2Y_{12}$ receptor (44% identity) and the fact that it is activated by a nucleotide (although of a different flavor), this receptor was brought into the P2Y receptor family three years after its identification (Abbracchio et al., 2003). The $P2Y_{14}$ receptor is found in a cluster of P2Y receptor genes encoding $P2Y_{12}$, $P2Y_{13}$, and a related receptor, GPR87, in a small region of chromosome 3 (q24-q25)(Fig. 1B). These genes share ~50% sequence identity and are just downstream of another P2Y receptor, the $P2Y_1$ receptor. As with the other two receptors in this cluster ($P2Y_{12}$ and $P2Y_{13}$), the $P2Y_{14}$ receptor is coupled to Gi, thereby resulting in inhibition of adenylate cyclase and potentially phospholipase C and GIRK channel activation. The mRNA for this receptor is widely expressed in humans with high levels found in neuronal tissue, placenta, adipose tissue, intestine, stomach and skeletal muscle, and lower levels in the lung, heart, spleen and pituitary tissues. Recently, activation of $P2Y_{14}$ receptors has been shown to mediate chemotaxis of bone-marrow hematopoietic stem cells, demonstrating a potential role in the immunomodulatory response (Lee et al., 2003a).

1.3I Misidentified 'P2Y' Receptors

In the race to identify novel P2Y receptors, several GPCRs were misidentified as purinergic receptors either by not realizing that a receptor was a homologue of an existing mammalian receptor or based on equivocal data/poor scientific method. One of the first examples of this misidentification was in the cloning of a receptor that was preferentially activated by nucleotide disphosphates and consequently identified as the novel '$P2Y_3$' receptor (Li et al., 1998; Webb et al., 1996). However, careful genetic and pharmacological analyses two years later accurately identified the $P2Y_3$ receptor as the avian homologue of the mammalian $P2Y_6$ receptor (Li et al., 1998). A more recent example includes a receptor that utilizes α-ketoglutarate as an agonist that was originally identified as the adenosine/AMP-activated $P2Y_{15}$ receptor (He et al., 2004; Inbe et al., 2004). This unfortunate misidentification occurred because the cells utilized in the study expressed endogenous receptors for adenosine as well as endogenous P2Y receptors (Qi et al., 2004). Other misidentified proteins include the lysophosphatidic acid (LPA) 4 receptor that was originally thought to be the $P2Y_9$ receptor as well as the $P2Y_7$ receptor, which is now known to be activated by leukotrienes and not nucleotides (Noguchi et al., 2003; Yokomizo et al., 1997). In addition, some orphan receptors that were identified as P2 receptors ($P2Y_5$, $P2Y_8$ and $P2Y_{10}$) have regained their orphan status upon further scientific investigation (Adrian et al., 2000; Li et al., 1997). Taken together, these 'missteps' by numerous investigators have left the P2Y receptor nomenclature with gaps that confound scientists to this day.

2. Regulation of P2 Receptor Signaling by Nucleotidases

Termination of nucleotide signaling is mediated by a large family of cell-surface nucleotide ectoenzymes, known as nucleotidases, that are as widely expressed as P2 receptors. More specifically, these enzymes regulate purinergic signaling by catalyzing the breakdown and interconversion of nucleotides at the cell-surface, thus modulating the concentration and species of nucleotide available in the extra-cellular space for P2 receptor activation. In 2000, a unifying nomenclature for a subset of these ectoenzymes was established that described members of the family as enzymes that can hydrolyze a single phosphate group from nucleoside 5'triphosphates and nucleoside 5'diphosphates with varying preference, thus, representing ecto-nucleoside 5'-triphosphate diphosphohydrolases (E-NTPDases) (Zimmermann, 2000). In addition, all members of the family harbor five conserved sequence domains known as the 'apyrase conserved regions'. Currently six members of the NTPDase family (NTPD1-6) have been identified and characterized, with individual members being divided into two subgroups based on predicted membrane topology. The first subgroup (NTPDase1-4) contains a large extracellular loop flanked by two transmembrane domains followed by either the intracellularly located N- or C-termini, while the second subgroup (NTPD5 and 6) is predicted to have an intracellular N-terminus followed by a single transmembrane domain and large extracellular domain that ends with the C-terminus. In both cases, the conserved 'apyrase regions' are located in the large extracellular domains. There are numerous published reports demonstrating the role NTPDases play in the regulation of P2 receptor signaling. In one example, expression of both NTPDase1 and 2 along with the $P2Y_1$ receptor resulted in an increased EC_{50} for varying

nucleotides at the receptor, thus, suggesting that pharmacological responses by P2YRs are dependent upon the subtypes of NTPDases present (Alvarado-Castillo et al., 2005).

Another family of nucleotidases that catalyzes alkaline phosphodiesterase and nucleotide pyrophosphatase activity is known as ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) enzymes. Members of this family include E-NPP 1-3 with their primary sequence predicting a membrane-bound structure with an intracellular amino terminus followed by a single transmembrane domain and a large extracellular domain. These enzymes are capable of hydrolyzing cAMP to AMP, ATP to AMP and PP_i, and ADP to AMP and P_i (Zimmermann, 2000). Furthermore, they are capable of hydrolyzing the pyrophosphate linkages of nucleotide sugars.

Other nucleotidases of note include the alkaline phosphatases, which are located on the extracellular leaflet of the plasma membrane and are fixed to the cell through a GPI-anchor. These are ecto-phosphomonoesterases with a broad substrate specificity that degrade nucleoside 5'-tri-, di- and –monophosphates. In addition, the ecto-5'-nucleotidase, which is attached to the the plasma membrane by a glycerolphosphatidylinositol (GPI) anchor, catalyzes the hydrolysis of nucleoside 5'-monophosphates to their respective nucleosides and P_i.

3. Role of P2Y Receptors in Epithelial Cells

Although P2Y receptors regulate multiple physiological processes in a variety of cells and tissues, one of their major roles is in the regulation of ion transport and stress response in epithelial cells (Insel et al., 2001; Leipziger, 2003). Epithelial cells line the interstitial surfaces in the lung, kidney, and intestine and create a barrier between the external

environment and the underlying cells and tissue. This paracellular barrier is created by a complex of proteins known as the tight junction, which forms an intercellular connection that creates a monolayer impermeable to water and ions. Tight junctions also serve to demarcate two distinct membrane surfaces in polarized epithelial cells: the apical surface, which lies above the tight junction and faces the lumen, and the basolateral surface, which lies below the tight junction and contacts underlying cells (Fig. 2). The differential expression of membrane proteins, including P2Y receptors, at 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 (Yeaman et al., 1999).

G protein-coupled P2Y receptors serve an important role in autocrine and paracrine regulation of ion and nutrient transport in epithelial cells. The first indication that P2Y receptors served in this capacity was the observation that ATP and UTP, when added to the apical surface of airway cells, promoted a Ca^{2+}-activated Cl^- current (Mason et al., 1991). Multiple subsequent studies showed that all five subtypes in the $P2Y_1$ receptor family are expressed in epithelial cells from various tissues (Cressman et al., 1999; Homolya et al., 1999; Leipziger, 2003; Marcus and Scofield, 2001; Post et al., 1998; Robaye et al., 2003; Wong and Ko, 2002; Zambon et al., 2001; Zambon et al., 2000). Moreover, many of these epithelial cells express multiple subtypes of P2Y receptors (Post et al., 1996). These studies demonstrate that all five Gq-coupled subtypes of P2Y receptors (and potentially the Gi-coupled subtypes) are expressed in epithelial cells and highlight the prominent role of extracellular nucleotides in regulation of epithelial cell function. In contrast, the $P2Y_{13}$ receptor does not appear to be expressed in epithelial cells, and although mRNAs encoding

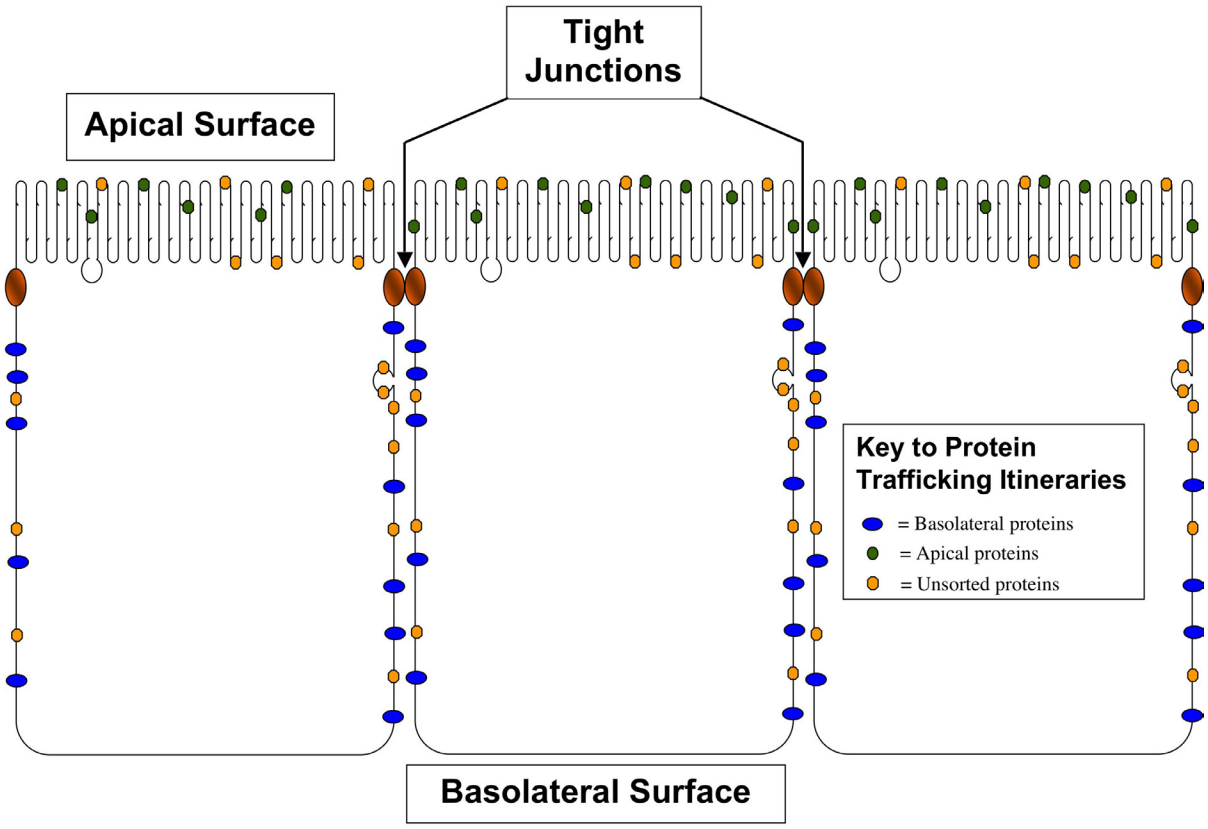


Figure 2. Topology of a Polarized Epithelial Cell Monolayer.

The key features of a polarized epithelial monolayer include two distinct membrane surfaces: the apical membrane which faces the lumenal space and the basolateral membrane which faces other cells and the underlying connective tissue. These two domains are separated by a protein complex known as the tight junction, which plays a critical role in establishing and maintaining cell polarity. One of the hallmarks of cell polarization is the delivery of proteins to either the apical or basolateral membrane domain. It is this segregation of proteins that allow epithelial cells to carry out a variety of specialized functions.

$P2Y_{12}$ and $P2Y_{14}$ receptors have been observed in tissues containing epithelial cells, direct demonstration of receptor expression in epithelial cells has not been reported.

Many of the aforementioned studies suggested that P2Y receptors are localized to distinct membrane surfaces in polarized epithelial cells. However, with the exception of the canine $P2Y_{11}$ receptor, in which a receptor-GFP fusion protein was shown to be targeted to the basolateral membrane of MDCK cells (Zambon et al., 2001), most of these studies have addressed the question of P2Y receptor polarization in an indirect manner or with potentially non-specific antibodies that lend uncertainty to the conclusions regarding polarized targeting.

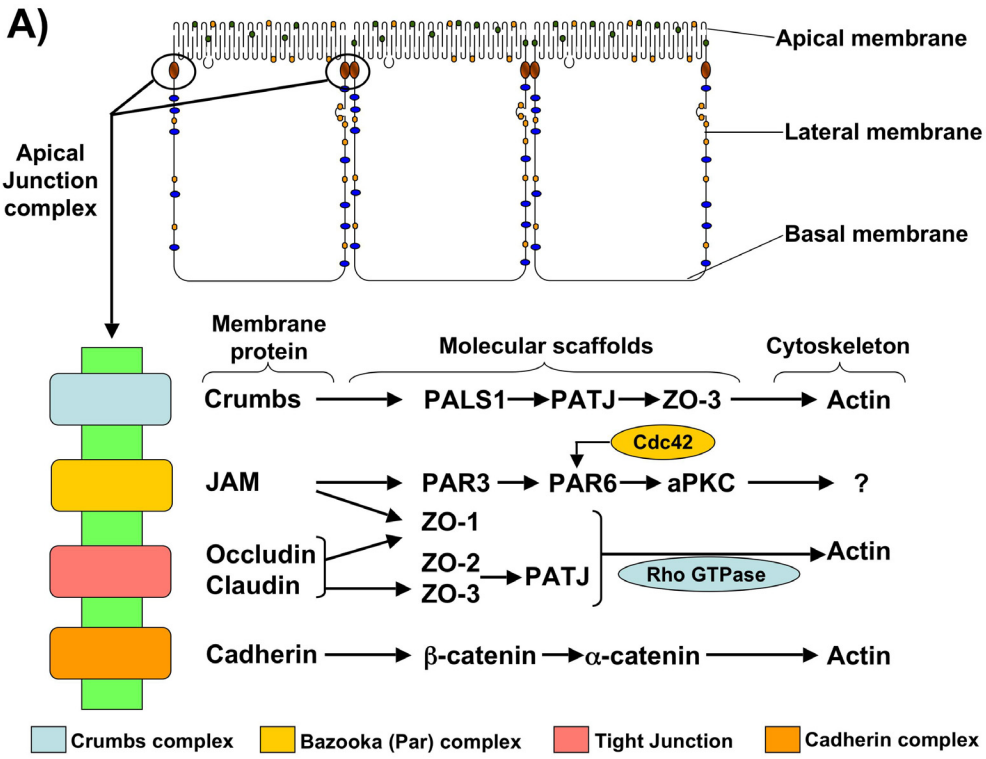
In order to fully appreciate targeting studies and the cellular context in which they take place, a basic understanding of how epithelial cells establish and maintain polarity is required. In the next few sections of this introduction, the process of epithelial polarization (i.e. proteins involved, required physical and spatial cues) will be described in some detail and should aid the reader in their understanding of this most elegant and complex process.

4. Physical, Spatial and Molecular Cues for Epithelial Cell Polarity

The complexity of multicellular tissues that contain more than one membrane domain requires numerous mechanisms to distinguish these domains as well as target and organize different proteins in each domain, and ultimately to keep the identities of the domains separate. It has taken the effort of numerous laboratories over a period of several decades to characterize this well-orchestrated and incredibly complex process (Nelson, 2003); thus, any attempt to describe this elegant process in great detail is beyond the scope of this chapter. Therefore, only the most important proteins and biochemical events required for establishing apical-basolateral polarity in epithelia will be presented.

The establishment and maintenance of cell polarity is a fundamental process controlling the behavior of all eukaryotic cells. Much has been learned about how epithelial cells utilize physical, spatial and molecular cues in order to generate the crucial apicobasal axis that is key to its specialized functions. Single epithelial cells in suspension culture exhibit non-polarized distributions of marker proteins that are differentially expressed at either the apical and basolateral membrane in monolayers. In order for these single epithelial cells to generate polarity, physical cues in the form of cell-cell and/or cell-substratum contact must occur. Once these extracellular contacts are made proteins are segregated into one of two distinct membrane surfaces: those in contact with cells/substratum or non-contacting (free) membrane surfaces facing the extracellular space. These contacting and non-contacting membrane surfaces (along with their unique proteins) are the respective precursors to the basolateral and apical membrane domains. In addition, the contact or lack thereof between these membrane surfaces puts into motion unique signaling cascades and mechanisms that begin to organize the cells into a polarized monolayer (Yeaman et al., 1999).

Initial cell-cell and/or cell-substratum contact commences the genesis of a crucial protein structure known as the apical junction complex (AJC), which is a multifunctional, modular structure containing several protein sub-complexes. Furthermore, each protein sub-complex comprises an integral membrane protein bound to scaffold proteins that are anchored in place through linkage to the actin cytoskeleton. Moreover, these protein sub-complexes typically contain many protein-protein interaction domains that facilitates their binding to other sub-complexes, all of which serves to put in motion the many signaling cascades required to establish a polarized epithelium (Fig 3A). The importance of the AJC is



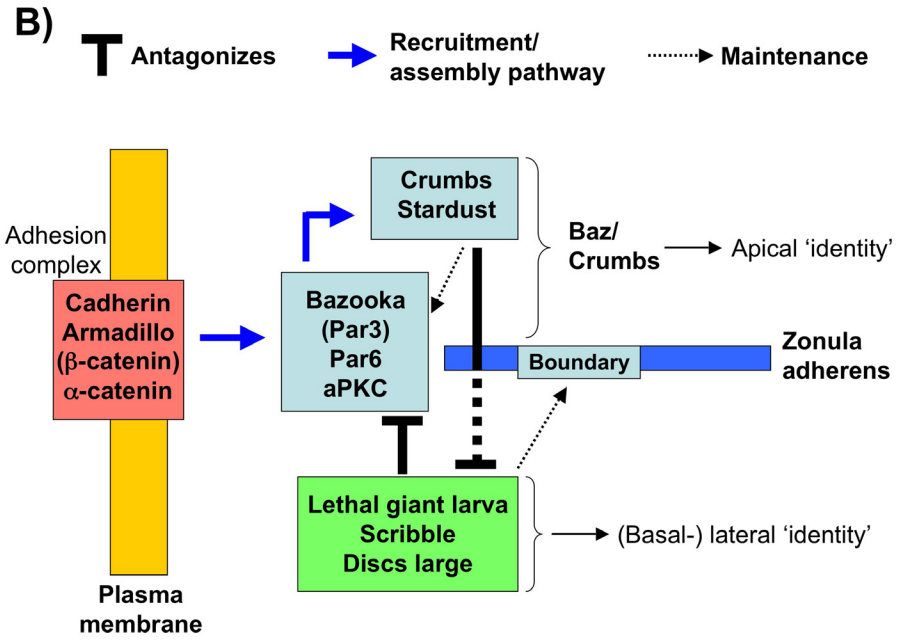


Figure 3. Organization of polarized epithelial cells and the apical junctional complex.

A) Polarized epithelial cells form a monolayer in which the apical membrane is separated at the boundary with the basal and lateral membranes by the apical junctional complex (AJC). The AJC is separated into structurally and functionally different sub-domains comprising

membrane proteins (Crumbs, JAM (junctional adhesion molecule) occluding/claudin and cadherin) linked to modular protein scaffolds, which in turn bind to the actin cytoskeleton. **B)** This is a simplified scheme for how different protein complexes in the apical junctional complex regulate cell-cell adhesion (cadherin complex) as well as apical membrane (Bazooka and Crumbs complex) and lateral membrane (Lethal giant larvae complex) formation. See text for details. This figure is loosely based upon a review by W.J. Nelson (Nelson, 2003).

reflected by the fact that it is highly conserved from *C. elegans* to mammals, with mutations to any one of its protein constituents usually causing an aberration in the establishment of the apicobasal axis and/or protein targeting. Finally, polarized epithelial cells form a monolayer in which the apical (free) surface is separated by the AJC at the boundary with the basal and lateral (bound) surfaces.

Genetic experiments carried out in both *C. elegans* and *Drosophila* models have identified key genes in the generation of epithelial cell polarity. For example, the genetic analysis of asymmetric cell division in *C. elegans* development led to the identification of seven proteins (partitioning-defective protein [PAR]1-6 and atypical protein kinase C [aPKC]) as key components of the molecular machinery required to generate cell polarity. More specifically, it was shown that Par3 (Bazooka), Par6 and aPKC proteins form a highly regulated sub-complex that is key to the formation of epithelial polarization (Etienne-Manneville and Hall, 2003). This is just one of the many major sub-protein complexes that comprises the AJC. Other sub-complexes include cadherin/catenin (α and β) proteins that constitute the adhesion complex, Crumbs (Crb)/PATJ/Stardust, Scribble (Scrib)/Discs Large (Dlg)/Lethal Giant Larvae (Lgl) proteins, and the zona occludens (ZO-1, ZO-2 and ZO-3), claudins and occludin subunits, which comprise the tight junction complex. Studies have shown that each of the protein complexes described above function in a regulated, common pathway that instructs the formation of the apical and lateral membranes.

The initial contact between cells leads to the formation of the adhesion complex, which acts a recruiter for several other protein complexes that instruct the formation of the apical and (baso-) lateral membrane domains. Upon recruitment to the adhesion complex, the Bazooka (Par6-aPKC-Par3) complex plays a key role in establishing polarity. The formation

and activation of this complex requires phosphorylation of Par6. Once activated, the Bazooka complex facilitates the formation of tight junctions, which are absolutely essential for the establishment and maintenance of epithelial cell polarity. More specifically, studies have demonstrated that the Par3 subunit of Bazooka interacts with the ZO-1 protein of tight junctions through an intermediary known as the junctional adhesion molecule (JAM) (Itoh et al., 2001) (Fig. 3A). These data suggest that movement of the activated Bazooka to the adhesion complex co-recruits components of the tight junction to the AJC by association with JAM. In addition to this co-recruiting activity, Bazooka seems to initiate the formation of the apical membrane. The Scrib/Dlg/Lgl complex, which is located below Bazooka, inhibits the spread of the apical membrane and maintains the lateral membrane identity by antagonizing both the Bazooka and Crb (Crumb/PATJ/Stardust) complexes. For its part, Crb is recruited apically to the Bazooka complex where it serves to maintain the identity of the apical membrane by antagonizing the activity the Scrib/Dlg/Lgl complex, which is to promote the spread of the lateral membrane (Fig 3B). In conclusion, these three complexes work in concert to establish and maintain the unique apical and lateral domain surfaces that are key to epithelial cell polarity.

4.1 Role of the cytoskeleton in epithelial cell polarity

The organization of the cell cytoskeleton (composed of actin and microtubles) plays a major role in the establishment and regulation of epithelial cell polarity. The actin cytoskeleton localizes to the cell cortex of each membrane domain with actin re-organization occurring in a bundle circumscribing the cell at the AJC where it attaches to the cadherin subunit of this large complex. In addition, the actin-based cytoskeleton forms the core of long

membrane protrusions, called microvilli, at the apical membrane of epithelial cells. Microtubles in polarized epithelial cells, such as MDCK cells, are organized in an apical web and in longitudinal bundles that run along the apical-basal axis of the columnar cell with their minus and plus ends located at the apical and baslateral membranes, respectively (Bacallao et al., 1989). Motor proteins specific to either the actin or microtuble cytoskeleton have been implicated in vesicle transport (discussed in more detail in Section 5.5 of this chapter). Generally, it is thought that the actin cytoskeleton is important for the local delivery of vesicles to a specific membrane domain, while microtubles seem to provide long-range transport for vesicles along the apicobasal axis.

5. Protein Sorting in Epithelial Cells

Once the polarity of epithelial cells is established, this critical property must be maintained in order to carry out many specialized functions including vectorial movement of ions and solutes. The cell employs an elaborate sorting process that utilizes complex machinery comprised of numerous molecules and utilizes several layers of regulation to create a high-fidelity system that ensures accurate delivery of biosynthetic cargo to either the apical or basolateral membrane domain. In fact, the mis-sorting of proteins in epithelia leads to certain pathologies that have been labeled as 'sorting' diseases. An example of this type of disease is cystic fibrosis, a condition caused by the failure to deliver the CFTR, an important ion channel, to the apical membrane of airway epithelia. This non-delivery of the CFTR to the luminal domain leads to improper hydration of lung epithelia, subsequent thickening of the mucous, and ultimately to chronic infections that eventually prove fatal.

Newly synthesized proteins that are destined for various compartments of the cell emerge from the trans-golgi network (TGN) and are delivered to their intended location. There are three routes of delivery for newly-synthesized proteins emerging from the Golgi/TGN that are destined to be delivered to a specific plasma membrane domain: direct delivery, transcytosis, and general delivery with selective retention (Fig. 4). Examples for all three routes of delivery have been described in numerous epithelial cell lines including MDCK(II) cells. In the simplest description, direct delivery of membrane-bound proteins involves the insertion of these nascent proteins into vesicles bound specifically for either the apical or basolateral membrane domain. Once loaded with a variety of proteins bound for the plasma membrane (PM), these vesicles are transported along the cell's cytoskeleton, utilizing a process dependent on small GTPases, protein complexes such as the exocyst and other chaperons. Upon reaching their intended location, the vesicles fuse with the plasma membrane and its cargo (membrane-bound proteins) are successfully delivered. However, much evidence has been presented that suggests the process may be more complex and convoluted. For example, vesicles bound for the basolateral membrane may first be delivered to the recycling endosome before reaching their final destination of the PM. In addition, recent work by Polishcuck *et al* (Polishchuk et al., 2004) suggests that the proteins destined for both the apical and basolateral are deposited in the same protein trafficking vesicles upon exiting the TGN and are later segregated at the level of the endosomes. Ongoing work by several laboratories will hopefully elucidate the exact path taken by proteins in order to establish and maintain the signature asymmetrical polarity of epithelia.

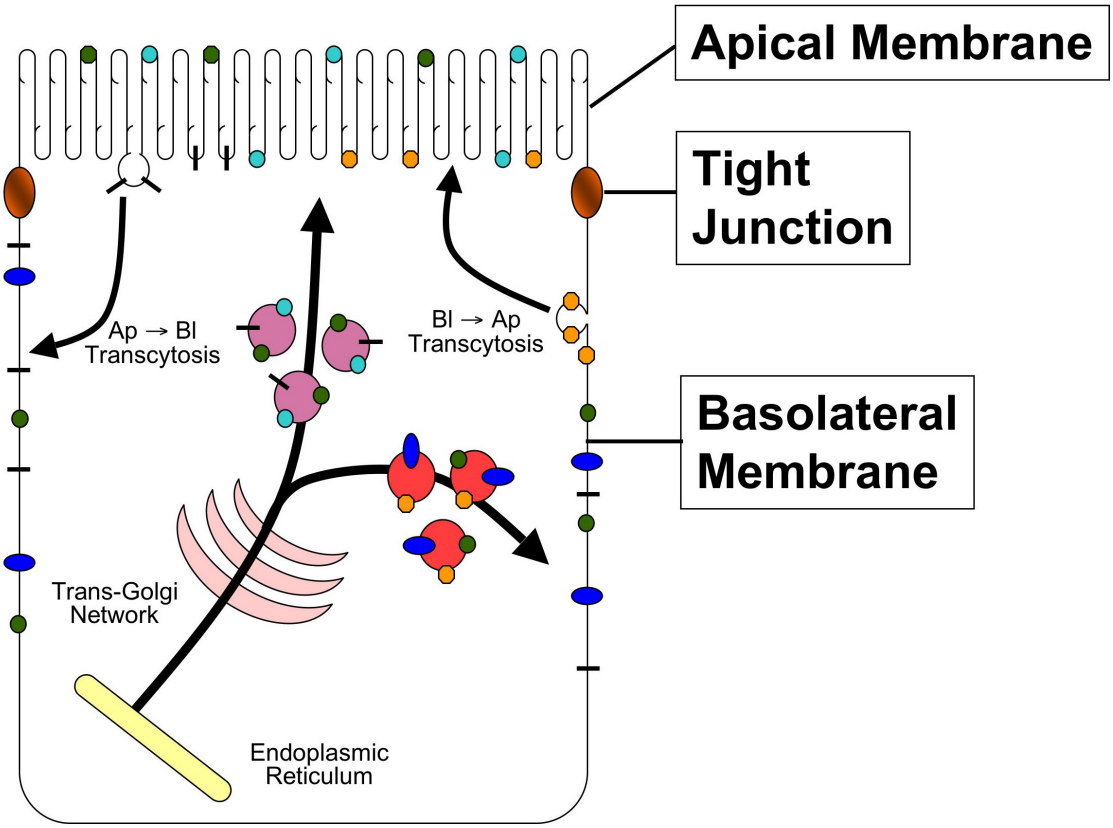


Figure 4. Protein Targeting in Polarized Epithelial Cells.

Newly synthesized proteins that are destined for various compartments of the cell emerge from the trans-golgi network (TGN) and are delivered to their intended location. There are three routes of delivery for newly-synthesized proteins emerging from the Golgi/TGN that are destined to be delivered to a specific plasma membrane domain: direct delivery, transcytosis, and general delivery with selective retention. Furthermore, the tight junction serves as a protein fence that prevents the mixing of apical and/or basolateral bound proteins.

The second mechanism employed by epithelial cells to ensure proper delivery of a subset of proteins is transcytosis. This mechanism of delivery involves sending a protein to either the apical or basolateral domain via vesicle trafficking and fusion with the plasma membrane, followed by the repackaging of the protein into a new vesicle that is then delivered to its final destination at the opposite membrane domain. An example of this mechanism of protein delivery is utilized by the apically expressed polymeric immunoglobulin (pIgR) receptor (Rojas and Apodaca, 2002). The pIgR is first delivered to the basolateral membrane, endocytosed and passes through a series endosomes before being delivered to the apical membrane. This type of transport is not often observed in MDCK(II) epithelial cells, but is more commonplace in hepatocytes and intestinal cells (Mostov et al., 2000).

Delivery of a protein to both the apical and basolateral membrane domain with its subsequent retention at only one of the membrane surfaces is the third mechanism utilized by epithelial cells to ensure polarized targeting. This mechanism is utilized by the cell in the case of the CFTR (Swiatecka-Urban et al., 2002), which contains a PDZ-binding motif that acts to stabilize the protein at the PM by interacting with a scaffolding protein. Typically, scaffold proteins contain several PDZ domains and proteins requiring anchoring and/or stability interacts with these scaffolds through their PDZ-binding motifs.

5.1 Protein kinase D (PKD) Regulates Protein Sorting

Protein kinase D (PKD) is a cytosolic serine-threonine kinase that binds to the TGN in a diacylglycerol-dependent manner and provides important regulation in the fission of transport vesicles destined for the cell surface (Baron and Malhotra, 2002). Experiments

carried out by Malhotra and colleagues have demonstrated that the active conformations of different PKD isoforms regulate the formation of transport vesicles within the TGN as well as their delivery to the basolateral membrane of MDCK(II) epithelial cells (Yeaman et al., 2004). In particular, they show that MDCK(II) epithelial cells endogenously express all three isoforms of PKD (1, 2, and 3), with each isoform promoting distinct effects on cargo transport from the TGN. Specifically, PKD1 and PKD2 are involved in the transport of basolateral, but not apical, cargo, while it is speculated that in polarized cells PKD3 regulates the exit of cargo from the TGN. The regulation of transport vesicle fission from the TGN by PKD lends fidelity to a very critical step in the biogenesis and maintenance of the basolateral membrane domain.

5.2 Involvement of the Exocyst in Protein Targeting

Once transport vesicles bound for the basolateral membrane leave the TGN, they transition to a new set of molecular machinery that ensures correct delivery to this specific membrane domain. One such machinery set is the exocyst, a well-studied and characterized protein complex that is localized to the lateral membrane just below the tight junction. This is a large multi-protein complex that includes Sec3, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 and is conserved from yeast to mammals, demonstrating the importance of this complex to cell function. In mammals, the exocyst (also known as the Sec6-Sec8 complex) is responsible for the delivery of a subset of proteins to the basolateral membrane in a highly regulated manner to maintain the fidelity of the cell's protein-sorting system. For example, this protein complex was demonstrated to be important for the basolateral targeting of LDL receptors (Grindstaff et al., 1998). Interestingly, the recruitment of the exocyst to the

basolateral membrane is enhanced by the expression of AP-1B, with co-localization data linking these two proteins in a transport complex (Folsch et al., 2003). These data suggest that basolateral targeting of membrane proteins (e.g. LDL and Tfn receptors) that interact with AP-1B may also depend on the exocyst as well.

Several small GTPases, including the Ral proteins, have been shown to be critical regulators of the exocyst. In a study published by Moskalenko *et al*, it was demonstrated that Ral proteins interact directly with the Sec5 subunit of the exocyst and promote its assembly. Moreover, perturbation of Ral expression caused disruption of basolateral, but not apical, targeting of plasma membrane proteins in polarized epithelial cells. (Moskalenko et al., 2002). In addition, Rab8 and Rab10 have also been shown to regulate the targeting of proteins to the basolateral membrane. In the case of Rab8, published experiments demonstrated that this small GTPase regulates basolateral targeting in an AP-1B-dependent manner (Ang et al., 2003). It was observed that expression of a mutant Rab8 protein disrupted the delivery of AP-1B dependent basolateral cargo and not basolateral proteins containing di-leucine-targeting motifs, which are known not to interact with AP-1B. Finally, it was demonstrated recently that both Rab8 and Rab10 most likely work in concert to regulate basolateral targeting (Schuck et al., 2007).

5.3 The Role of Cdc42 in Protein Sorting

Cdc42 is a Rho-family GTPase that regulates the transport of proteins to the basolateral membrane of epithelial cells (e.g. MDCK). In a landmark study by Mellman and colleagues, deletion of Cdc42 from MDCK cells resulted in the selective depolarization of basolateral proteins, while the polarity of apical proteins remained unaffected (Kroschewski

et al., 1999). Subsequent studies demonstrated that Cdc42 regulates the delivery of membrane-bound, but not soluble, proteins to the basolateral domain through modulation of the actin cytoskeleton by an as-yet unidentified mechanism (Cohen et al., 2001; Musch et al., 2001). Interestingly, the expression of a dominant-negative Cdc42 protein in MDCK cells caused disruption in targeting of basolateral proteins that depend on AP-1B for their delivery, thus implicating Cdc42 as one of many modulators of the exocyst (Ang et al., 2003).

5.4 SNAP/Syntaxin role in targeting

A set of machinery utilized by virtually all cells to ensure vesicles dock and fuse with the correct membrane domain is the conserved SNARE (soluble N-ethylmaleimide-sensitive factor [NSF] attachment protein [SNAP] receptor) machinery. The SNARE hypothesis describes a universal model for the fusion of vesicles with intracellular membranes, such as the plasma membrane, and has been the focus of numerous laboratories. This fusion machinery consists of integral membrane proteins on the vesicle (v-SNAREs) and on the target membrane (t-SNAREs) that interact during the process of vesicle docking. In mammalian cells, t-SNAREs include members of the syntaxin and SNAP-25 families while v-SNAREs include members of the VAMP/synaptobrevin family. Moreover, there are several soluble proteins such as NSF and α-SNAP that interact with the v- and t-SNAREs, which are thought to be critical for the overall membrane traffic process. The SNARE hypothesis originally postulated that this machinery plays a major role in the specificity of membrane fusion by correctly pairing members of the large v-SNARE family with corresponding members of the large t-SNARE family. Indeed, it has been shown that different isoforms of v-SNAREs and t-SNAREs interact in different membrane traffic

pathways in order to achieve distinct subcellular localizations of the vesicular protein cargo (Low et al., 1998a).

Recognizing that subcellular localization of SNARE isoforms could provide additional fidelity to the protein sorting machinery in MDCK(II) epithelial cells, Mostov and co-workers investigated the role of the SNARE machinery in polarized membrane trafficking (Low et al., 1996). Their initial studies in MDCK(II) cells demonstrated that members of the t-SNARE family, syntaxins 2, 3 and 4, were endogenously expressed in this cell line with a polarized distribution for two of them. Syntaxins 3 and 4 were respectively localized to the apical and basolateral membrane domains, while syntaxin 2 was present at both membrane domains. Follow up studies concluded that TGN-to-apical transport does indeed utilize the apical-specific syntaxin 3 as well as α-SNAP and SNAP-23, while TGN-to-basolateral transport likely utilizes the basolateral-specific syntaxin 4 along with synaptobrevin/VAMP-2, NSF, α-SNAP and SNAP-23 (Low et al., 1998b). The authors speculated that in addition to MDCK(II) epithelial cells, all other epithelial cell lines utilize specific members of the SNARE machinery for membrane fusion, although to what degree is unknown.

5.5 Role of Cytoskeletal Motors in Targeting

The internal cytoskeleton of the cell provides an important structure that is absolutely required in establishing and maintaining epithelial cell polarity. This critical structure is composed of microtubules organized into bundles aligned along the apicobasal axis with the plus end at the basal pole and the minus end at the apical pole. In addition, actin microfilaments form the core of long membrane protrusions at the apical membrane, known as microvilli, as well as forming a ring around the apex of the lateral membrane (Yeaman et

al., 1999). Motor proteins specific for both microtubles and actin microfilaments have been characterized and shown to be involved in the transport of proteins to the plasma membrane. More specifically, these motors interact with transport vesicles in an ATP-dependent manner, allowing them to ferry proteins along the cytoskeleton to a specific plasma membrane domain.

Transport of vesicles along microtubules is mediated by dyneins for movement toward the minus end of the microtubules, while kinesins mediate movement toward the plus end of microtubules. A very large and diverse family myosin motor proteins mediate transport along the actin cytoskeleton (Berg et al., 2001). Both families of motor proteins have been implicated as mediators of polarized protein targeting. In the case of microtubule motor proteins, dynein was shown to interact with the C-tail of the rhodopsin GPCR in order to ferry the receptor along microtubules to the apical membrane of MDCK epithelial cells (Tai et al., 1999). The perturbation of the delivery of numerous proteins in polarized epithelial cells by colchicine treatment, which disrupts microtubules, underscores the role of microtubules and their cognate motor proteins in the delivery of transport vesicles to specific membrane domains. For example, colchicine treatment disrupts apical targeting of aquaporin protein 2 (AQP2) (Brown, 2003; Sabolic et al., 1995). Myosin-based delivery of membrane proteins has also been demonstrated to play a major role in protein targeting, with class V myosins mediating transport in epithelial cells (Rodriguez and Cheney, 2000). For example, myosin-Vc was shown to be involved in the membrane trafficking of the transferrin receptor. Finally, Rab proteins of the small GTPase family have been recognized as important modulators of motor-mediated protein delivery, with over 60 different mammalian members

each regulating a distinct intracellular transport step of this important process (Bock et al., 2001; Jordens et al., 2005).

5.6 Targeting Signals in Polarized Proteins

Proteins with a polarized distribution contain a targeting signal that is read by the sorting machinery of the cell to ensure proper delivery to either the apical or basolateral membrane domain. Targeting signals are usually part of a protein's primary sequence and vary in length from only a few amino acids to more than 20 residues. In addition to short motifs, post-translational modifications including N- and O-linked glycosylation and GPI anchors have been shown to act as apical targeting signals, while secondary protein structure such as the β-turn can act as both an apical (Sun et al., 2003) and basolateral targeting signal (Reich et al., 1996). Apical and basolateral targeting signals are discussed in more detail below.

6. Apical Targeting Signals

In contrast to BL targeting signals (see below), identification of sorting signals for apically bound proteins has proven to be more elusive. One reason for the difficulty in identifying apical targeting signals may be that these signals can be located in any of the different domains of membrane proteins. For example, protein-based apical targeting sequences are located in extracellular domains, as is the case for $P2Y_2$ (Qi et al., 2005) and $P2Y_6$ receptors (A.-D. Qi et al., unpublished results), transmembrane (TM) domains, e.g. the 4^{th} transmembrane domain of the gastric H^+-ATPase (Dunbar et al., 2000), and intracellular domains, e.g. the Bile Acid Transporter and Vitamin C transporter (Subramanian et al., 2004; Sun et al., 2001). Proteins containing a covalently linked GPI modification are sorted to the

apical membrane of many (but not all) polarized epithelial cell types (Brown et al., 1989; Lisanti et al., 1989). This lipid modification is thought to associate with membrane structures called lipid rafts, which are composed of clustered glycosphingolipids, cholesterol, and certain other proteins (Mostov et al., 2000). In addition, both N- and O-linked oligosaccharides have been suggested to act as apical sorting signals (Scheiffele et al., 1995; Yeaman et al., 1997), but there are many exceptions and thus the role of glycosylation as a sorting signal is unclear. Finally, a role of C-terminal PDZ-binding domains in localization of proteins to the apical surface is emerging. For example, the PDZ-binding domain of the CFTR is critical for its apical localization and its interaction with the PDZ domain-containing protein, EBP50 (Moyer et al., 1999; Moyer et al., 2000).

6.1 GPI Anchor and N- and O-linked glycosylation

Post-translational modifications of proteins play a role in their targeting to the apical membrane domain. For example, the addition of a GPI anchor to nascent proteins has been shown to promote their insertion into apically-bound lipid rafts in MDCK(II) epithelial cells (detailed in the next section) (Polishchuk et al., 2004), while the role of other modifications such as N or O-linked glycosylation is less clear. The role of carbohydrate moieties, such as glycosylation, as a targeting determinant was first identified in experiments that showed apically secreted proteins lacking their endogenous N-glycans were released in a non-polarized fashion in MDCK(II) cells. In addition, a role for N-glycans in apical targeting of membrane-bound proteins was clearly demonstrated for three different proteins in the context of polarized MDCK(II) epithelial cells (Gut et al., 1998). This evidence, as well as others, helped to establish a N-glycan sorting hypothesis that described an apical-targeting pathway

based upon an interaction between N-or O-glycans and specific TGN lectin-sorting proteins that promote incorporation of glycosylated proteins into vesicles bound for the apical membrane. However, evidence against the direct role of N-glycans as an apical targeting determinant also began to emerge, including studies showing that removal of N-linked glycosylation had no effect on polarized sorting (Marzolo et al., 1997; Yeaman et al., 1997).

In light of the conflicting evidence regarding N-glycans as an apical sorting determinant, an alternative model has been put forth in which this moiety has been assigned a more indirect role in apical targeting (Rodriguez-Boulan and Gonzalez, 1999). More specifically, the authors suggest that N-glycans facilitate apical targeting by stabilizing the targeting signal, thus allowing the modified protein to exit the TGN and be inserted into appropriate vesicles bound for the apical membrane. Evidence consistent with this hypothesis include the demonstration that N-glycosylation increases resistance to thermal denaturation and/or stabilizes a folded conformation (Wang et al., 1996). However, it should be noted that to date no unequivocal demonstration of an apical pathway based upon lectins interacting with N- or O-glycans has been reported.

6.2 Lipid rafts and apical targeting

The apical membrane mediates many of the specialized functions that are key to epithelial cells. Furthermore, this membrane faces a dynamic and hostile environment, which requires it to be quite durable. This durability is a function of the composition of the lipid membrane, which is a unique mixture of different lipids enriched in sphingolipids and cholesterol (Simons and van Meer, 1988). Because of the presence of these lipids, the apical membrane has a strong propensity to form tightly packed membrane microdomains called

lipid rafts (Schuck and Simons, 2004; Simons and Ikonen, 1997). The lipid raft hypothesis posits that rafts exist in the plasma membrane of most cells as small, highly dynamic, liquid-ordered assemblies that are embedded in a surrounding liquid-disordered membrane (Simons and Vaz, 2004). Moreover, it was discovered that these rafts are resistant to solubilization with mild detergents such as Triton X-100 and therefore are sometimes referred to as detergent-resistant membranes (DRMs) or detergent-insoluble glycosphingolipid-enriched membrane domains (DIGs). Once formed, lipid rafts serve as islands that bring together a variety of proteins that are important for cellular functions such as signal transduction (Simons and Toomre, 2000). Moreover, these rafts are very dynamic structures that are constantly being turned over and are regulated by a variety of proteins.

The apical membrane of epithelial cells, particularly in the kidney and intestine, has a large capacity for secretion and absorption and therefore requires tight control over the insertion of proteins in order to maintain these critical processes. For example, insertion of the wrong transport protein could potentially lead to the aberration of a steep ion gradient across the epithelial monolayer that is required for proper kidney function. This requirement for a high-fidelity sorting system relies on a variety of apical-targeting determinants (i.e. linear signals, glycosylation) including association with lipid rafts. The enrichment of sphingolipids and cholesterol at the apical membrane of epithelia make it an ideal place for the formation of lipid rafts and it has been hypothesized that rafts preferentially traffic to this particular membrane after intracellular assembly (Simons and Ikonen, 1997). More specifically, it is thought that the entire raft, with all of the associated proteins, are assembled at the level of TGN and delivered ‘wholesale’ to the apical membrane. The insertion of a subset of proteins into apically-bound lipid rafts has been shown to be regulated by a post-

translation modification known as a glycosylphophatidylinositol (GPI) anchor. For example, the addition of this anchor to the placental alkaline phosphatase (PLAP) acts as an apical determinant by allowing it to associate with lipid rafts (Brown and Rose, 1992). More recently, Polishchuck and colleagues applied a combination of live-cell imaging and fluorescent labeling technologies to provide further evidence that the GPI-proteins are delivered to the apical membrane of MDCK(II) cells by association with lipid rafts (Polishchuk et al., 2004).

6.3 Linear Sequences direct Apical Targeting

One of the first insights into the nature of apical targeting came from studies that demonstrated certain post-translational modifications, such as GPI moieties, acted as signals that ensured delivery of proteins to the apical membrane (Fiedler et al., 1993; Le Gall et al., 1995). However, subsequent published studies demonstrated that distinct, linear sequences located in the extracellular, transmembrane and intracellular domains of proteins are necessary and sufficient to confer apical targeting (Cheng et al., 2002; Chuang and Sung, 1998; Hodson et al., 2006; Qi et al., 2005; Takeda et al., 2003). Moreover, these targeting signals vary in length from a short peptide of a few amino acids ($P2Y_2$ receptor) to an almost entire C-terminus of 39 amino acids (rhodopsin receptor). Upon inspection of this collection of apical targeting signals, one notices it is quite diverse with seemingly little in common. In fact, some of these published apical targeting signals were analyzed for shared motifs in order to establish an apical targeting motif (Cheng et al., 2002), but did not yield any results. Unfortunately, the elucidation of linear apical targeting signals is several years behind the basolateral counterpart and requires further investigation to advance this particular subfield

of protein targeting research. A recent spate of apical targeting studies is encouraging and should begin to elucidate common apical targeting motifs.

7. Basolateral Targeting Signals

Sorting signals for basolaterally targeted proteins are usually short, cytoplasmic sequences that can be grouped into one of three classes. The first class is characterized by a short motif containing an essential tyrosine residue either as a part of an NPXY motif (where X is any amino acid) or a YXXΦ motif (where Φ is a bulky hydrophobic residue), while the second class can be defined by two amino acids, i.e. a di-hydrophobic (typically di-leucine) motif. The third class is composed of a heterologous mixture of motifs that vary in length and demonstrates no similarities to well-characterized basolateral-targeting signals.

Originally, the targeting of molecules to the apical membrane was thought to occur by an active process comprised of protein-based signals that ensured entry into the apical sorting machinery, while proteins lacking such signals were by default delivered to the basolateral membrane (Mostov et al., 1986). However, descriptions of short amino-acid motifs that were necessary and sufficient to confer basolateral targeting onto unsorted proteins (Aroeti et al., 1993; Hermosilla and Schulein, 2001; Mostov et al., 2000) demonstrated that targeting machinery for the basolateral membrane exists as well. Basolateral targeting signals are typically short amino-acid motifs located within the cytoplasmic domain of the targeted protein. The best-characterized signal is the four amino acid tyrosine-based motif that not only is sufficient to direct targeting to the basolateral membrane, but has been shown to interact with adaptor proteins (AP) to mediate endocytosis (Mostov et al., 1999). The duality of this signal suggests an alternative pathway for protein trafficking exists wherein proteins

move from the TGN to a common/sorting endosome first and then are delivered to a specific membrane compartment. Another well-characterized sorting signal is the di-leucine motif, which has been shown to deliver a number of proteins to the basolateral membrane (Rodriguez-Boulan et al., 2005). Finally, secondary protein structure has been proposed to be important in the function of basolateral targeting signals. For example, the NPXY motif found in the pIgG receptor protein has been shown to adopt a beta-turn structure that is thought to be critical for its polarized targeting (Aroeti et al., 1993).

7.1 AP proteins (1-4) involvement with BL sorting

Typically, linear sorting signals are recognized by cytosolic protein complexes that regulate delivery to a specific membrane domain. The adaptor protein (AP) family is one such complex. These are hetero-tetrameric complexes composed of two large subunits (γ, α, δ or ε and β1-β4), one medium subunit (μ1-μ4) and one small subunit (σ1-σ4). There are four major species of AP complexes: AP-1 (γ, β1, μ1, σ1), AP-2 (γ, β2, μ2, σ2), AP-3 (δ, β3, μ3, σ3), and AP-4 (ε, β4, μ4, σ4). Furthermore, there are two sub-species of the AP1 protein, AP-1A (γ, β1, μ1A, σ1) and AP-1B (γ, β1, μ1B, σ1), with the former being ubiquitiously expressed and the later being expressed only in a subset of epithelial cells (MDCK, Caco-2, HT-29, Hec-1-A and RL-95-2). AP-1, AP-3 and AP-4 facilitate sorting at the TGN or endosomes, while AP-2 acts at the plasma membrane to facilitate internalization (Folsch, 2005). Whereas the large subunits (γ, α, δ, ε and β1-β4) interact with a variety of accessory proteins, the medium subunits (μ1-μ4) interact directly with tyrosine-based targeting signals and therefore are responsible for cargo recognition.

In most cases, tyrosine-based basolateral targeting signals are recognized by the epithelial-specific variant of AP-1, AP-1B, which to date is the best-characterized protein complex for basolateral targeting. The role of AP-1B in targeting proteins to the basolateral membrane of epithelial cells was elucidated by Mellman and colleagues in their studies with LLC-PK1 epithelial cells, which are deficient for the μ1B subunit (Folsch et al., 1999). In these studies, LDL and transferrin receptors, which are normally targeted to the basolateral membrane of MDCK(II) cells, are mis-sorted to the apical membrane of LLC-PK1 cells. Furthermore, exogenous expression of μ1B in LLC-PK1 epithelia 'rescued' basolateral targeting of both receptor proteins, demonstrating that the tyrosine-based signals contained within these proteins interact with μ1B in order to achieve a polarized distribution.

More recently, AP-4 has been implicated in the basolateral targeting of several membrane proteins in MDCK(II) epithelial cells (Simmen et al., 2002). Hunziker and co-workers first established that all four AP species (1-4) are endogenously expressed in MDCK(II) epithelia and then specifically tested whether AP-4 binds different types of cytosolic signals known to mediate basolateral targeting. They demonstrated that AP-4 binds the di-hydrophobic signal in the furin protein, the tyrosine-based motif in the LDLR protein (but not the tyrosine-based motif in the transferrin receptor), and the unconventional signal in the MPR 46 protein. Furthermore, they show significant disruption in basolateral sorting of LDLR and MPR 46 proteins in μ4-depleted MDCK(II) cells. Taken together, these results strongly suggest that the medium-sized μ4 subunit of the AP-4 complex interacts with a subset of proteins through their cytosolic signals in order to promote basolateral targeting, which is similar to how the AP-1B complex functions as well. Since both adaptor complexes function in a similar manner, it is tempting to speculate that AP-4 is also regulated by small

GTPases and probably interacts with the same cell machinery such as the exocyst. Moreover, these two adaptor protein complexes may act in concert to generate and maintain polarity in epithelial cells.

8. Sorting signals in G protein-coupled receptors

Until recently, distinct sorting signals in G protein-coupled receptors (GPCRs) had not been identified. Much of the early work on identifying trafficking itineraries and targeting signals in GPCRs was carried out by Limbird and coworkers with α2-adrenergic receptors (Keefer and Limbird, 1993; Wozniak et al., 1997; Wozniak and Limbird, 1996; Wozniak and Limbird, 1998). These studies showed that three subtypes of α2-adrenergic receptors are delivered to the basolateral membrane by distinct targeting mechanisms that utilize multiple, non-contiguous targeting signals. In contrast to these results, more recent studies have identified linear targeting sequences in three different GPCRs. The cytoplasmic tail of rhodopsin was shown to act as a novel apical sorting signal in polarized MDCK cells (Chuang and Sung, 1998), while a 14 amino acid basolateral targeting sequence was identified in the C-terminal tail of the follicle stimulating (FSH) receptor (Beau et al., 1998). The 39 amino acid targeting signal for rhodopsin was shown to interact with dynein as the mechanism for apical targeting (Tai et al., 1999), while Tyr-684 and Leu-689 were found to be most important in determining basolateral targeting for the FSH receptor. Finally, a 21-amino acid sequence in the 3rd intracellular loop of the M_3-muscarinic receptor was shown to confer basolateral targeting (Nadler et al., 2001).

9. Hierarchy of Sorting Signals

The discovery of targeting signals and the related cellular machinery as the mechanism driving polarized delivery of proteins in epithelial cells did begin in earnest until the early 1980's. It was during this early period that several 'laws' of targeting were established based upon a few scientific studies, only to be discounted by contradictory follow-up studies. One such early 'law' was the notion that basolateral targeting was the default pathway for proteins lacking a targeting signal, while apically bound proteins were actively delivered via a sorting signal. This early law was debunked by studies demonstrating the existence of short peptide sequences that were necessary and sufficient to drive basolateral targeting (Mostov et al., 1986). More recently, it was suggested that the default pathway is the delivery of cell-surface proteins to both the apical and basolateral membrane domains, while polarized delivery is an active process requiring specific targeting signals and an elaborate set of machinery (Muth and Caplan, 2003).

During the course of scientific studies designed to elucidate targeting signals it was discovered that the removal of a primary sorting determinant resulted in the exposure or activation of a previously cryptic/secondary sorting determinant. For example, removal of the basolateral targeting signal from the follicle-stimulating hormone (FSH) receptor revealed a secondary apical targeting signal (Beau et al., 1998). Such results have led to the idea that there exists a hierarchy of sorting signals within polarized proteins for reasons unknown. Moreover, this led to another dogmatic rule that basolateral targeting signals always override apical targeting signals when located in the same protein. However, evidence to the contrary challenges this idea, including results reported in Chapter V of this dissertation. In the end,

the mechanisms that determine the hierarchy of multiple sorting signals in a single protein remain unclear.

10. Stability of proteins upon successful delivery

Actin-based scaffolding systems reside at both the apical and basolateral membrane domains of epithelial cells and provide an essential 'anchoring' platform for newly delivered membrane-bound proteins that lends them stability. Membrane-bound proteins interact with scaffolding complexes through a multitude of interacting proteins and domains that provides stability and co-localization with other proteins necessary for proper signal transduction. For example, the Na^+-K^+-ATPase binds both ankyrin and fodrin, which in turn interact with scaffolding complex located at the basolateral membrane of MDCK epithelial cells (Nelson and Hammerton, 1989). In another example, syntrophin interacts with utrophin at the basolateral membrane of epithelial cells in order to recruit and assemble a complex of proteins required at this membrane domain (Kachinsky et al., 1999).

One of the most common and well-characterized modular protein-interaction domains is the PDZ domain. First identified and described in the early 1990's, the name PDZ is derived from the proteins in which these domains were identified: PSD-95, DLG and ZO-1 (Cho et al., 1992; Kim et al., 1995; Woods and Bryant, 1993). Well over 75 proteins have been identified as 'PDZ-domain' proteins (Fanning and Anderson, 1999), which provide an essential organizational role for protein complexes at the plasma membrane. PDZ-domains form a binding pocket for proteins containing PDZ-binding motifs, which are usually quite short and found at the extreme C-terminus of a protein. Moreover, PDZ proteins often have multiple PDZ domains, allowing these proteins to have multiple binding partners, and

contain additional domains that allow them to interact with the actin cytoskeleton. Utilizing these multiple binding domains, PDZ-proteins serve as an organizational bridge between cell-surface proteins and the cytoskeleton. This bridging lends cell-surface proteins a great deal of stability and increased retention at both the apical and basolateral membrane. In addition to establishing and maintaining stability, PDZ-proteins serve to organize specific sets of proteins at the plasma membrane that in turn carry out important physiological functions. For example, the Na exchanger regulatory factor (NHERF) family has been shown to coordinate the formation of multiprotein complexes that modulate trafficking, transport and signaling in polarized epithelial cells (Thelin et al., 2005).

11. Rationale for Dissertation Research

Prior to the commencement of my dissertation research, numerous investigators had examined the role of P2Y receptors in regulation of epithelial cell physiology. These studies revealed that P2Y receptors show prominent and ubiquitous expression in many epithelial-containing tissues and mediate multiple physiological functions in epithelia, including the regulation of ion channels and the response to stress (Insel et al., 2001; Leipziger, 2003). In addition, results from many of these investigators suggested that several of the P2Y receptor responses were polarized (i.e. observed at either the apical or basolateral membrane, but not both). However, with the exception of a single study by Insel and colleagues (Zambon et al., 2001), none of these published reports demonstrated polarized expression patterns directly using methods such as immunofluorescence.

The possibility that multiple P2Y receptors were expressed in polarized manner in epithelial cells motivated us to examine the distribution of all the P2Y receptors in epithelial

cells in a direct manner. Indeed, our experiments revealed an interesting expression pattern for the P2Y receptor family in that seven of the eight members were targeted in a polarized manner (described in Chapter II). We hypothesized that P2Y receptors contained sorting signals that mediated targeting of these proteins to either the apical or basolateral membrane. In order to test this hypothesis, we constructed a series of chimeric P2Y receptors to localize potential targeting signals, and examined their localization in MDCK(II) epithelial cells by confocal microscopy. These experiments allowed us to identify and locate multiple targeting signals within the P2Y receptor family (described in Chapter III). Once the general location of all the P2Y receptor targeting signals had been elucidated, we delimited and characterized two of these signals: a basolateral targeting signal located in the $P2Y_1$ receptor and an apical targeting signal located in the $P2Y_4$ receptor (described in Chapters IV and V respectively).

CHAPTER II: Polarized expression of human P2Y receptors in epithelial cells from kidney, lung, and colon

1. Introduction

Extracellular nucleotides such as ATP and UTP are released from essentially all cells and interact with cell surface P2 receptors to produce a broad range of physiological responses. P2 receptors are divided into two major classes: P2X receptors, which are non-selective cation channels, and P2Y receptors, which are members of the G protein-coupled receptor (GPCR) superfamily (Harden, 1998; Ralevic and Burnstock, 1998). Molecular cloning and characterization studies have identified eight functional human G protein-coupled P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$), which fall into two subfamilies based on their signaling properties and sequence identities. The $P2Y_1$ receptor family, comprising $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors, activates heterotrimeric G proteins of the Gq family, thereby activating phospholipase C and promoting inositol lipid-dependent signaling (Harden, 1998; Ralevic and Burnstock, 1998). In addition to activating Gq, the $P2Y_{11}$ receptor also activates Gs and therefore stimulates adenylyl cyclase activity (Communi et al., 1997; Qi et al., 2001a; Torres et al., 2002). The $P2Y_{12}$ receptor subfamily, comprising $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors, activates $G_{i/o}$ thereby promoting inhibition of adenylyl cyclase activity (Chambers et al., 2000; Communi et al., 2001; Hollopeter et al., 2001; Zhang et al., 2002).

Although P2Y receptors regulate multiple physiological processes in a variety of cells and tissues, one of their major roles is in the regulation of ion transport and stress responses in epithelial cells (Insel et al., 2001; Leipziger, 2003). Epithelial cells line the interstitial surfaces in the lung, kidney, and intestine and create a barrier between the external environment and the underlying cells and tissue. This paracellular barrier is created by a complex of proteins known as the tight junction, which forms an intercellular connection that creates a monolayer impermeable to water and ions. Tight junctions also serve to demarcate two distinct membrane surfaces in polarized epithelial cells: the apical surface, which lies above the tight junction and faces the lumen, and the basolateral surface, which lies below the tight junction and contacts underlying cells. The differential expression of membrane proteins, including P2Y receptors, at 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 (Yeaman et al., 1999).

G protein-coupled P2Y receptors serve an important role in autocrine and paracrine regulation of ion and nutrient transport in epithelial cells. The first indication that P2Y receptors served in this capacity was the observation that ATP and UTP, when added to the apical surface of airway cells, promoted a Ca^{2+}-activated Cl^- current (Mason et al., 1991). Multiple subsequent studies showed that all five of the subtypes in the $P2Y_1$ receptor family are expressed in epithelial cells from various tissues (Cressman et al., 1999; Homolya et al., 1999; Leipziger, 2003; Marcus and Scofield, 2001; Post et al., 1998; Robaye et al., 2003; Wong and Ko, 2002; Zambon et al., 2001; Zambon et al., 2000). Moreover, many of these epithelial cells express multiple subtypes of P2Y receptors (Post et al., 1996). Although mRNAs encoding $P2Y_{12}$ and $P2Y_{14}$ receptors have been observed in tissues containing

epithelial cells, direct demonstration of receptor expression in epithelial cells has not been reported. These studies demonstrate that all five Gq-coupled subtypes of P2Y receptors (and potentially the Gi-coupled subtypes) are expressed in epithelial cells and highlight the prominent role of extracellular nucleotides in regulation of epithelial cell function.

Many of the aforementioned studies suggested that P2Y receptors are localized to distinct membrane surfaces in polarized epithelial cells. However, with the exception of the canine $P2Y_{11}$ receptor, in which a receptor-GFP fusion protein was shown to be targeted to the basolateral membrane of MDCK cells (Zambon et al., 2001), most of these studies have addressed the question of P2Y receptor polarization in an indirect manner or with potentially non-specific antibodies that lend uncertainty to the conclusions regarding polarized targeting. To avoid these mitigating factors and to define the targeting properties of the entire family of P2Y receptors in epithelial cells, we determined the steady-state localization of P2Y receptors by visualizing HA-tagged receptors expressed in MDCK(II) cells by confocal microscopy. These studies were further supplemented by quantification of receptor distribution by biotinylation and measurement of agonist-induced changes in short-circuit current. Remarkably, our data indicate that all but one of the eight P2Y receptors are localized exclusively to either the apical or basolateral membrane surfaces of MDCK(II) cells. Moreover, a nearly identical targeting profile of the Gq-coupled P2Y receptor family 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. This is the first study to define the targeting properties of the entire family of P2Y receptors in polarized epithelial cells.

2. Materials and methods

2.1 Construction of HA-tagged receptor constructs

Human P2Y receptor cDNA constructs were amplified by PCR with *Pfu* polymerase (Stratagene, La Jolla, CA) from Hela cell genomic DNA. The upstream primers were complementary to codons 2-8 of each individual P2Y receptor, while the downstream primers were reverse complementary to the last 6 codons and the stop codon of each coding sequence. To aid in subcloning, the 5' primers contained an *Mlu*I site immediately preceding the second codon of the individual receptor, while the 3' primers contained an *Xho*I site following the stop codon. The 5' primer for the $P2Y_{11}$ receptor, which is the only P2Y receptor whose coding sequence is interrupted by an intron, comprised an *Mlu*I site, codons 2-4 from the first exon and codons 5-11 of the second exon (Communi et al., 1997). The amplified cDNAs were digested with *Mlu*I and *Xho*I and ligated in-frame into a similarly digested pLXSN retroviral expression vector containing a Kozak initiation sequence (ACCATGG), initiating methionine residue, and the hemagglutinin (HA) epitope tag (YPYDVPDYA).

2.2 Cell culture and expression of receptor constructs

Madin-Darby canine kidney type II cells (MDCK(II); ATCC, Rockville, Maryland) were subcultured in DMEM/F12 (1:1) medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS; Hyclone, Gaithersburg, MD) and 1X pen/strep in a humidified incubator at 37^{o}C with 5% CO_2 and 95% air. 16HBE14o- cells, an immortalized human bronchial epithelial cell line, were grown (Cozens et al., 1994) on collagen-coated

plates in MEM medium (Invitrogen) supplemented with 10% FBS, 1% sodium pyruvate, 1% non-essential amino acids and 1X penicillin/streptomycin. CaCo-2 cells (Fogh et al., 1977), an immortalized human colonic epithelial cell line, were grown in the same medium as 16HBE14o- cells, except that the FBS concentration was increased to 20%.

Recombinant retroviral particles were produced by calcium phosphate-mediated transfection of PA317 cells with pLXSN vectors containing HA-tagged hP2Y receptor constructs as previously described (Comstock et al., 1997) and used to infect the cell lines listed above. Geneticin-resistant cells were selected after 7-10 days with 1 mg/ml G418 and maintained in medium containing 0.4 mg/ml G418.

2.3 Confocal microscopy

MDCK(II), 16HBE14o- and CaCo-2 cells stably expressing HA-tagged hP2Y receptor constructs were seeded at a density of 6 x 10^5 cells/well in 12 mm polyester Transwell inserts (0.4 mM; Corning Life Sciences, Acton, MA). All cells were allowed to polarize for 7 days with daily medium changes. Cell monolayers were washed with cold PBS^{++} (phosphate-buffered saline containing 10 mM Ca^{2+} and Mg^{2+}), fixed and permeabilized with –20^o C methanol for 4 min, and blocked with PBS containing 1% non-fat dry milk for 30 min at room temperature. Receptors were labeled with anti-HA mouse monoclonal antibody HA.11 (Covance, Berkeley, CA) and tight junctions were labeled with a rabbit polyclonal antibody to ZO-1 (Zymed, South San Francisco, CA). Cells were washed 3 times with cold PBS^{++} and labeled with goat anti-mouse A-488 (for P2Y receptors) and goat anti-rabbit A-594 (for ZO-1) secondary antibodies (Molecular Probes, Eugene, OR). The fixed and stained monolayers were washed several times with cold PBS^{++}, excised from

the Transwell inserts, and mounted on glass microscope slides with Slowfade mounting media (Molecular Probes).

Confocal microscopy was performed on an Olympus Fluoview 300 laser scanning confocal imaging system (Melville, NY) configured with an IX70 fluorescence microscope fitted with a PlanApo 60X oil objective. Multiple XY (horizontal to the monolayer) and XZ (vertical to the monolayer) scans were acquired for each monolayer.

2.4 Quantification of cell-surface HA-tagged P2Y receptors.

MDCK(II) cells stably expressing HA-tagged hP2Y receptors were seeded in duplicate in 24 mm Transwell inserts and allowed to polarize as described above. Monolayers were placed on ice and kept at 4^{o}C for the duration of the experiment. Cells were washed with cold PBS^{++} three times for five min each, and then labeled with 1 mg/ml Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL) in cold PBS^{++} buffer, pH 8, for 40 min. The biotin solution was removed and the reaction quenched with 100 mM glycine in PBS^{++} for 10 min. The cells were washed and then incubated for 5 min with 0.7 ml Tris-Triton buffer (50 mM Tris•HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing a protease inhibitor cocktail. The cells were passed 7-10 times through a 25-gauge needle and then incubated for 1.5 h with rocking. The cell lysate was centrifuged at 20,000 x *g* for 30 min and the supernatant incubated with 50 ml of immobilized Neutravidin (Pierce) for 1.5 h. The resin was washed twice with Tris-Triton buffer, and biotin-labeled proteins were eluted from the Neutravidin resin by incubating with 35 ml of SDS-PAGE sample buffer containing 100 mM dithiothreitol for 10 min at 37^{o}C. The dithiothreitol cleaves the disulfide within the biotin spacer and releases the proteins from Neutravidin under mild conditions.

Eluted proteins were separated by SDS-PAGE on a 10% gel and transferred overnight to nitrocellulose membranes. Membranes were blotted via a standard western blotting protocol with the anti-HA mAb conjugated to HRP (3F10; Roche Biochemicals, Indianapolis, IN). The blots were developed with SuperSignal West Pico chemiluminescent substrate (Pierce) and the resulting bands were imaged on a BioRad Flour-S system and quantitated with BioRad QuantityOne software (BioRad, Hercules, CA).

2.5 Radioligand Binding Assay

A binding assay for membranes was performed as previously described (Waldo et al., 2002). Briefly, various concentrations of MDCK(II) membranes were incubated with an approximate K_i concentration (8 nM) of [^{3}H]MRS2279 for 30 minutes at 4^{o}C. Binding reactions were terminated by the addition of 4 mL cold assay buffer (20 mM Tris•HCl, pH 7.5, 145 mM NaCl, 5 mM $MgCl_2$) and filtered through GF/A filters to retain membrane-bound [^{3}H]MRS2279. Filters were washed once with cold assay buffer and placed in scintillation fluid for measurement of radioactivity. Specific binding of 8 nM [^{3}H]MRS2279 to MDCK membranes was defined as total radioligand bound minus the radioligand bound in the presence of 30 mM MRS2179, a $P2Y_1$ receptor-selective antagonist (Jacobson et al., 1998).

2.6 Ussing chamber measurement of short circuit current

MDCK(II) cells stably expressing HA-tagged hP2Y receptors were seeded in 12 mm polyester Snapwell inserts (Corning Life Sciences, Acton, MA) and allowed to polarize for 5-7 days as described above. The inserts were placed in Ussing chambers and monitored for

changes in short circuit currents (I_{SC}) in response to cumulative concentrations of the appropriate nucleotides added to either the mucosal (apical) or serosal (basolateral) surface. The maximal response at each concentration was plotted as a cumulative increase in I_{SC} versus nucleotide concentration.

3. Results

3.1 Localization of HA-tagged P2Y receptors in MDCK(II) cells

To determine the membrane targeting properties of the entire family of P2Y receptors in epithelial cells, we individually expressed HA-tagged constructs of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors in MDCK(II) cells by retroviral infection and determined the steady-state localization of each receptor in polarized monolayers by confocal microscopy. MDCK(II) cells have been used extensively as a model cell line to define the targeting properties of a broad range of membrane proteins (Keefer and Limbird, 1993; Mostov et al., 2000; Nadler et al., 2001). MDCK(II) cells expressing each P2Y receptor were cultured in transwells and allowed to polarize for 7 days. The cells were then fixed and labeled with antibodies directed against either the HA epitope or ZO-1 (a marker of the epithelial tight junction) as described in Materials and Methods.

Figure 5 shows XY and XZ cross-sections of wild-type MDCK(II) cells and MDCK(II) cells expressing each of the eight P2Y receptor subtypes. Wild-type MDCK(II)

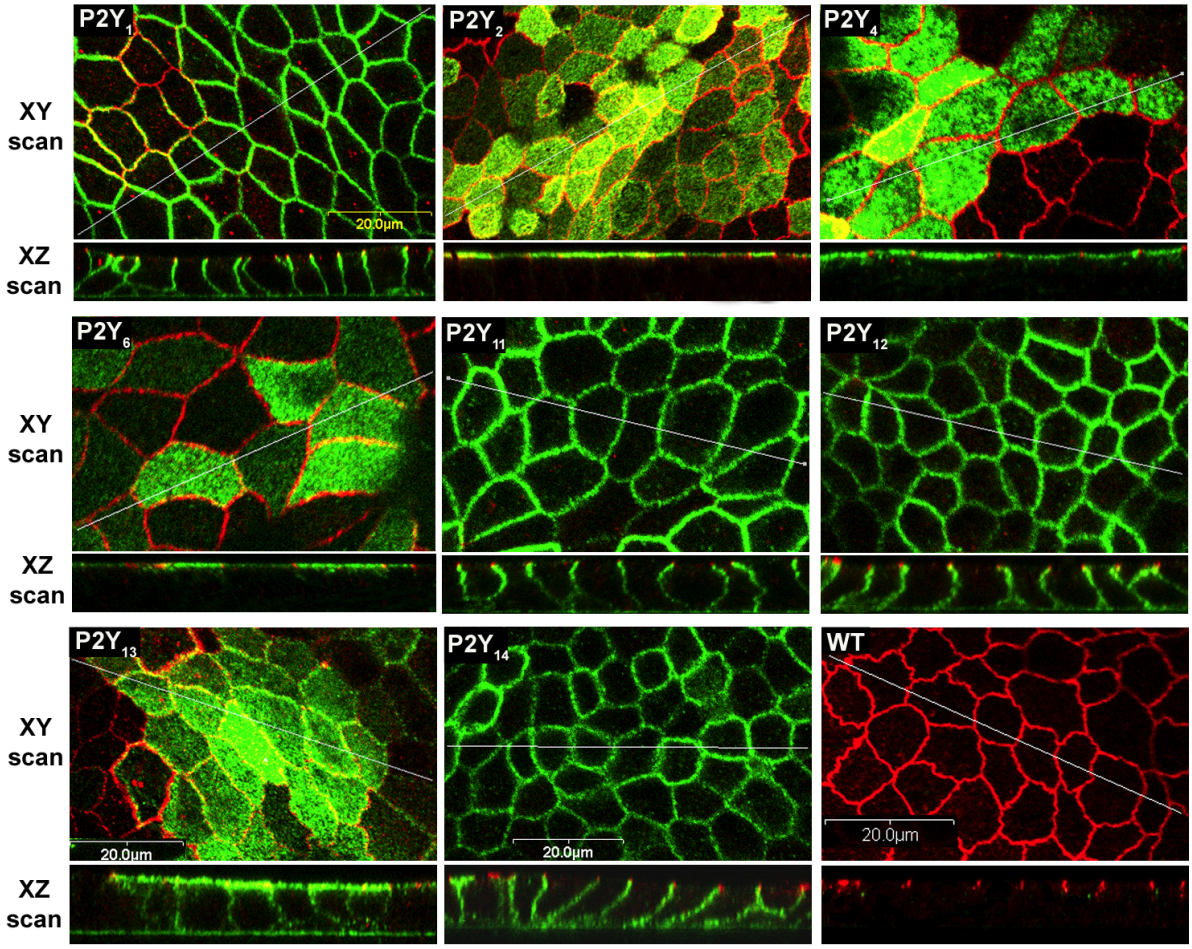


Figure 5. Confocal microscopy of wild-type MDCK(II) cells and MDCK(II) cells expressing HA-tagged hP2Y receptors.

MDCK(II) epithelial cells expressing each of the HA-tagged P2Y receptor subtypes and wild type MDCK(II) cells (WT) were examined by confocal microscopy. For each cell line, the top panel is a confocal image in which the focus plane was parallel to the monolayer (XY scan), while in the bottom panel the focus plane was a vertical cross-section of the monolayer (XZ scan). The white line in the XY scan indicates the path of the XZ scan. Green fluorescence represents HA-tagged P2Y receptor and red fluorescence represents the ZO-1 subunit of the tight-junction protein complex. The lack of green fluorescence in WT MDCK(II) cells demonstrates the specificity of the anti-HA antibody.

cells showed staining of the tight-junctions but no staining with the anti-HA antibody, demonstrating the specificity of both antibodies in MDCK(II) cells. Confocal micrographs of MDCK(II) cells expressing each P2Y receptor revealed that seven of the eight receptor subtypes were localized at steady-state to either the apical or basolateral membrane surface. $P2Y_1$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{14}$ receptors were expressed heavily along the lateral regions of the cell below the tight junction with a low level of expression at the basal membrane. Essentially no visible staining for these receptors was observed in the apical membrane. In contrast, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors were expressed exclusively at the apical membrane, with little to no staining below the tight junction (Fig. 5). The only receptor that was not localized was the $P2Y_{13}$ receptor. Thus, the family of P2Y receptors shows a distinct pattern of polarized expression in MDCK(II) cells.

3.2 Biotinylation of P2Y receptors in MDCK(II) cells

To provide a more quantitative measure of receptor polarization, we utilized a biotinylation assay to determine the levels of receptor expression at either the apical or basolateral membrane. MDCK(II) cells expressing each P2Y receptor were biotinylated from either the apical or basolateral surface, and biotinylated receptors were quantified as described in Materials and Methods. Representative blots are shown in Figure 6 and the percentages of biotinylated receptors at each membrane surface are presented in Table 1. Consistent with the confocal images, all of the receptor subtypes except the $P2Y_{13}$ receptor showed a strongly polarized steady-state localization, with $\geq$ 96% of $P2Y_1$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{14}$ receptors expressed on the basolateral surface and $\geq$ 97% of the $P2Y_2$, $P2Y_4$, and

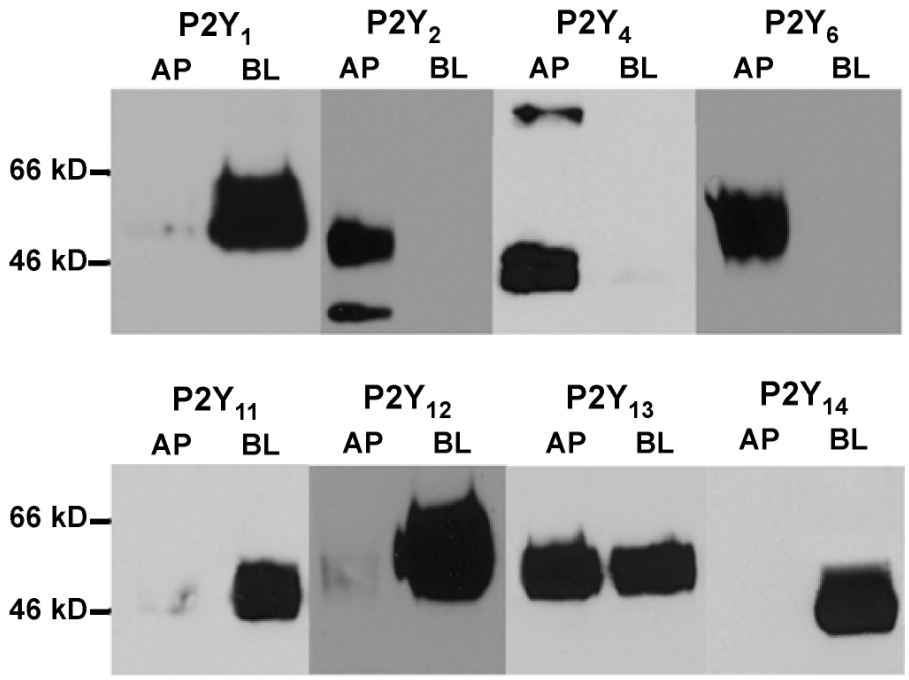


Figure 6. Representative western blots of biotinylation studies on HA-tagged P2Y receptors expressed in MDCK(II) epithelial cells.

The apical or basolateral membrane surfaces of MDCK(II) cells expressing each of the HA-tagged P2Y receptors were biotinylated, the biotinylated proteins were bound to neutravidin resin, and the percentage of biotin-labeled receptors at each membrane surface was quantified by Western blotting with anti-HA antibody. Western blots for each of the eight P2Y receptor subtypes are representative of at least three independent experiments. The results for the quantification of the western blots are shown in Table 1.

Receptor	% Expression	
	Apical	Basolateral
$P2Y_1$	1 ± 0.3	99 ± 0.1
$P2Y_2$	97 ± 2	3 ± 2
$P2Y_4$	98 ± 1	2 ± 1
$P2Y_6$	100	0
$P2Y_{11}$	4 ± 2	96 ± 2
$P2Y_{12}$	2 ± 2	98 ± 2
$P2Y_{13}$	54 ± 1	46 ± 1
$P2Y_{14}$	1 ± 0.3	99 ± 0.3

Table 1. Quantification of cell-surface expression of P2Y receptors.
Cell-surface expression of P2Y receptors at the apical and basolateral membrane surfaces was quantified by a polarized biotinylation assay. P2Y receptor-expressing MDCK(II) cells were labeled with biotin from either the apical or basolateral surface, and labeled receptors were quantitated as described in Materials and Methods. Values ± standard errors were averaged over three experiments

$P2Y_6$ receptors expressed on the apical surface. The $P2Y_{13}$ receptor was expressed at similar levels at both membrane surfaces.

3.3 Localization of the $P2Y_1$ receptor subfamily in 16HBE14o- and CaCo-2 cells

To confirm that polarized expression of P2Y receptors in MDCK(II) cells is not a cell-specific phenomenon, we also expressed HA-tagged $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors in two immortalized human epithelial cell lines, 16HBE14o- bronchial epithelial cells (Abraham et al., 2004; Cozens et al., 1994; Forbes, 2000) and CaCo-2 colonic adenocarcinoma cells. As in MDCK(II) cells, $P2Y_1$ and $P2Y_{11}$ receptors were expressed at the basolateral surface in 16HBE14o- and CaCo-2 cells, while $P2Y_4$ and $P2Y_6$ receptors were expressed at the apical membrane (Fig. 7). The $P2Y_2$ receptor was expressed at the apical membrane in both 16HBE14o- and CaCo-2 cells, but in 16HBE14o- cells the receptor also was expressed at lower levels in the lateral membrane below the tight junctions (Fig. 7). These data demonstrate that the Gq-coupled P2Y receptors are targeted in an essentially identical manner in epithelial cells derived from three distinct tissues, suggesting that targeting of the Gq-coupled P2Y receptor subtypes is not dependent on the type of epithelial cell in which they are expressed.

3.4 Functional expression of P2Y receptors in MDCK(II) cells

Progress in molecular and physiological studies of the P2Y receptors has been hampered by the lack of reliable radioligands for their quantification. However, Harden and

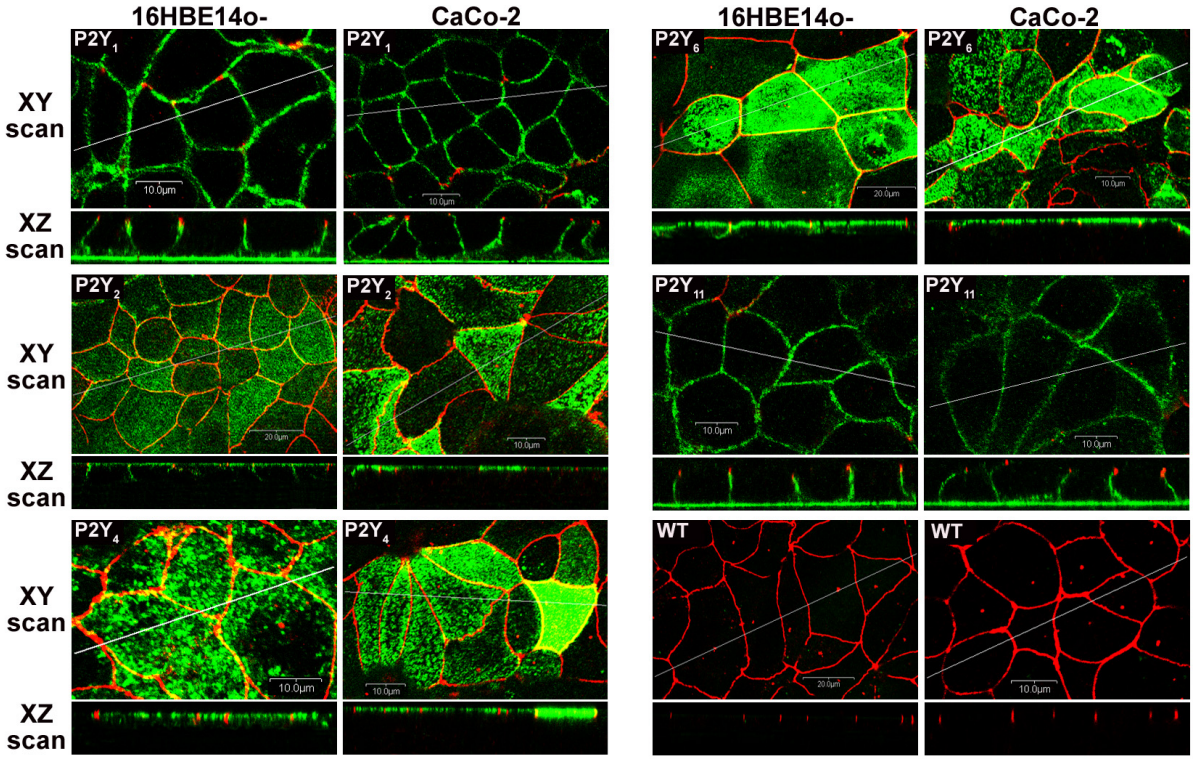


Figure 7. Confocal microscopy of HA-tagged Gq-coupled hP2Y receptors in 16HBE14o- and CaCo-2 epithelial cell lines.

HA-tagged $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors were expressed individually in bronchial 16HBE14o- and colonic CaCo-2 epithelial cell lines and the targeting of each receptor was determined by confocal microscopy as described in the legend to Figure 5.

co-workers (Waldo et al., 2002) recently developed [^{3}H]MRS2279 as a radioligand that is effective for quantification of natively and exogenously expressed $P2Y_1$ receptors. This radioligand was utilized to quantify $P2Y_1$ receptors in wild type MDCK(II) cells and after stable expression of the human $P2Y_1$ receptor. $P2Y_1$ receptor levels increased from 8 ± 1 fmol/mg protein for the endogenous canine $P2Y_1$ receptor of wild type MDCK(II) cells to 185 ± 5 fmol/mg protein after selection of a population of MDCK cells stably expressing the exogenous human $P2Y_1$ receptor.

The functional activity of the exogenous $P2Y_1$ receptor also was examined in polarized MDCK(II) cells by measuring I_{SC} in Ussing chambers across monolayers of wild type cells or cells expressing the $P2Y_1$ receptor (Fig. 8). I_{SC} is the summation of the flow of both cations and anions through multiple channels across a monolayer of cells. Increasing concentrations of 2MeSADP were added cumulatively to either the apical or basolateral compartments and I_{SC} was measured. Interpretation of these experiments was complicated by the endogenous expression in MDCK cells of the canine homologues of $P2Y_1$, $P2Y_2$ and $P2Y_{11}$ receptors (Post et al., 1998; Zambon et al., 2001), which give rise to increases in I_{SC} in the absence of exogenous expression of human P2Y receptors. Thus, we relied on the observation that concentration-response curves for agonists shift to the left as a function of increases in GPCR expression (Kenakin, 1997). This procedure has been utilized previously by Zambon et al. in their studies with the canine $P2Y_{11}$ receptor (Zambon et al., 2001). Consistent with our confocal and biotinylation experiments, overexpression of the $P2Y_1$ receptor in MDCK(II) cells promoted a 12-fold leftward shift in the concentration-response curve for 2MeSADP at the basolateral membrane (Fig 8, Table 2). Interestingly, this shift to

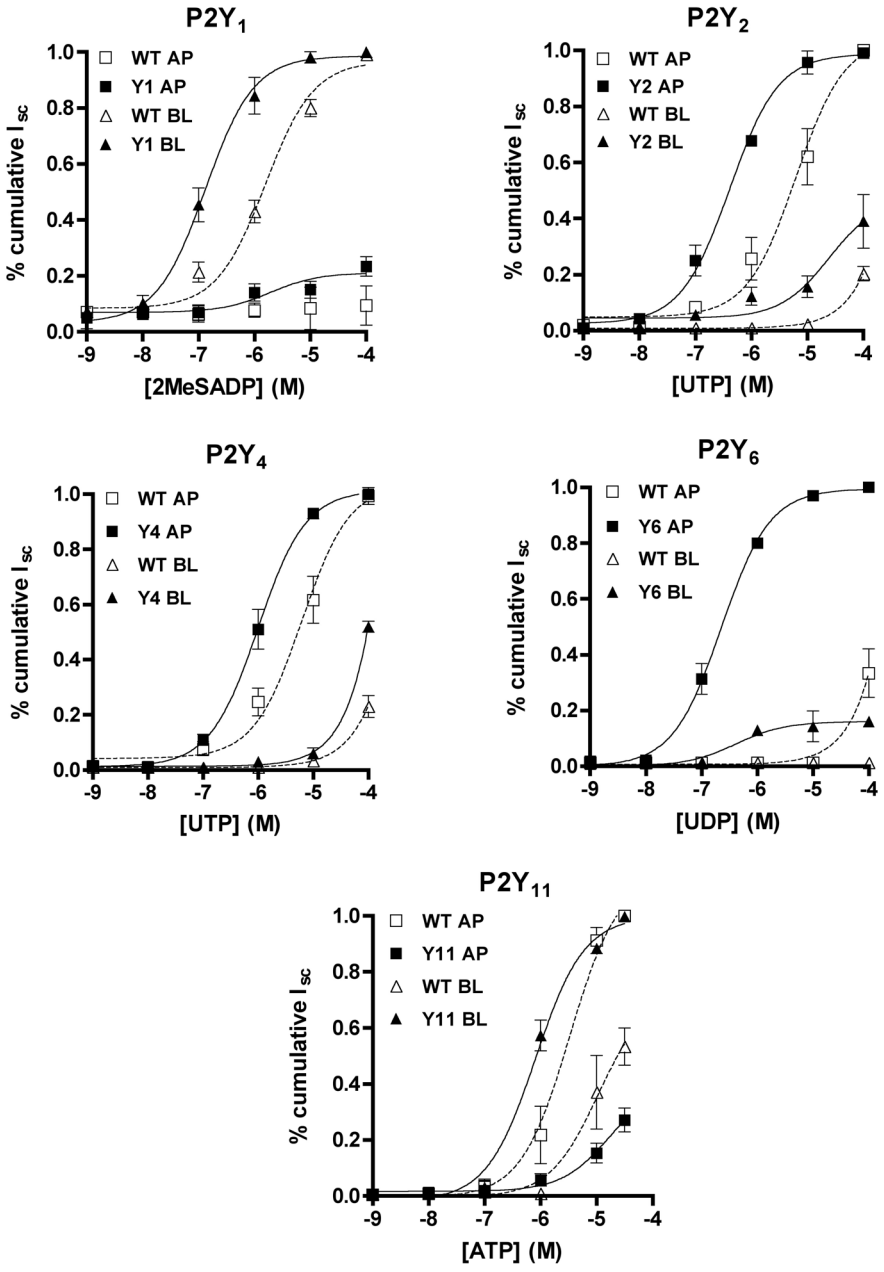


Figure 8. Measurement of I_{sc} in wild-type and P2Y receptor-expressing MDCK(II) cells.

Wild type and P2Y receptor-expressing MDCK(II) monolayers were challenged with cumulative doses of the appropriate nucleotides in Ussing chambers and the resulting I_{sc} was measured. Short-circuit currents in response to increasing concentrations of the indicated nucleotide are plotted for both wild-type and P2Y receptor-expressing MDCK(II) cells and are representative traces for three independent experiments. The EC_{50} values (μM) of the indicated nucleotides are shown in Table 2.

		EC_{50} Wild-type cells		EC_{50} P2Y-expressing cells	
Receptor	**Nucleotide**	**AP**[a]	**BL**[b]	**AP**	**BL**
$P2Y_1$	2MeSADP	NR[c]	1.6 ± 0.1	1.9 ± 0.3[e]	0.1 ± 0.1
$P2Y_2$	UTP	6.2 ± 0.1	ND[d]	0.4 ± 0.1	24 ± 0.2[f]
$P2Y_4$	UTP	6.2 ± 0.1	ND	1.0 ± 0.1	ND
$P2Y_6$	UDP	ND	NR	0.2 ± 0.1	0.4 ± 0.2[g]
$P2Y_{11}$	ATP	3.3 ± 0.1	ND	ND	0.8 ± 0.1

Table 2. EC_{50} values for P2Y regulation of I_{SC}.

Wild type and P2Y receptor-expressing MDCK(II) monolayers were challenged with increasing cumulative doses of the appropriate nucleotides. EC_{50} values (μM) are shown. [a]AP, apical; [b]BL, basolateral; [c]NR, no response; [d]ND, not determined (the curve did not reach a maximum); [e]relative efficacy of 0.2 compared to $P2Y_1$-BL; [f]relative efficacy of 0.5 compared to $P2Y_2$-AP; [g]relative efficacy of 0.2 compared to $P2Y_6$-AP.

the left in the concentration-response curve for 2MeSADP was similar to the approximately 20-fold increase in $P2Y_1$ receptor density quantified by [^{3}H]MRS2279 binding. Moreover, both the magnitude and potency of the 2MeSADP-promoted apical currents were considerably lower than those promoted by basolaterally applied 2MeSADP. The increase in I_{SC} following apical application of 2MeSADP in MDCK(II) cells expressing the human $P2Y_1$ receptor likely represents a small amount of expression of the receptor at the apical surface, again consistent with our confocal and biotinylation studies.

Similar results were obtained with the other Gq-coupled receptor-expressing cells. That is, overexpression of the P2Y receptor in MDCK(II) cells resulted in a leftward shift in the concentration-response curves for the appropriate agonists (UTP for $P2Y_2$- and $P2Y_4$-expressing cells, UDP for $P2Y_6$-expressing cells, and ATP for $P2Y_{11}$-expressing cells) at the membrane surface to which the receptor was primarily targeted (Fig. 8, Table 2). Unusual results were observed with the $P2Y_{11}$ receptor since the ATP response at the apical membrane of wild type MDCK(II) cells decreased significantly following expression of the $P2Y_{11}$ receptor. However, the basolateral ATP responses (where the $P2Y_{11}$ receptor is exclusively expressed; Fig. 5) were entirely consistent with those obtained with the other receptors. In contrast to the five Gq-coupled subtypes, we did not observe changes in I_{SC} in cells expressing Gi-coupled $P2Y_{12}$, $P2Y_{13}$, or $P2Y_{14}$ receptors, suggesting that Gi-mediated pathways are not involved in regulating epithelial I_{SC} under our experimental conditions.

4. Discussion

Although much work has been carried out on membrane protein localization in polarized epithelial cells, a relative paucity of data exists documenting the polarization of G

protein-coupled receptors. In the current study, we examined the polarization of the entire family of P2Y receptors, many of which are known to play important roles in the function and regulation of epithelial cells. Our data demonstrated that seven of the eight members of the family of P2Y receptors are expressed in a highly polarized manner in MDCK(II) epithelial cells, and these results were verified both in biotinylation studies and I_{sc} measurements in live cells. This is the first study to visualize directly the steady-state localization of the entire P2Y receptor family with confocal microscopy.

The marked polarized distribution of seven of the eight P2Y receptor subtypes was striking. In contrast, only two of the five muscarinic receptor subtypes, M2 and M3, are targeted to distinct membrane domains in MDCK(II) epithelial cells (Nadler et al., 2001). Polarization of P2Y receptors may result from the fact that all five of the Gq-coupled receptors (Homolya et al., 1999; Lazarowski et al., 1997b; Wong and Ko, 2002; Zambon et al., 2001) (and potentially two of the three Gi-coupled receptors (Chambers et al., 2000; Hollopeter et al., 2001)) are natively expressed in polarized cell types, i.e. epithelial and endothelial cells, where targeting of receptors to distinct membrane surfaces is critical for proper function. These data suggest that the seven polarized receptors contain targeting signals that direct the protein to either the apical or basolateral surface. Therefore, receptors not known to be expressed endogenously in epithelial cells, such as the $P2Y_{13}$ receptor, might lack the proper targeting information to ensure a polarized distribution and by default have an unsorted phenotype. Our data are consistent with this idea.

The targeting profile of the family of P2Y receptors revealed an unexpected pattern. P2Y receptors activated solely by adenine nucleotides, i.e. the $P2Y_1$, $P2Y_{11}$, and $P2Y_{12}$ receptors, are localized to the basolateral membrane of MDCK(II) epithelia, whereas those

P2Y receptors activated by uridine nucleotides, i.e. $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors, are localized to the apical membrane (Fig. 9). The $P2Y_{14}$ receptor, which is activated by UDP-sugars such as UDP-glucose, is also localized to the basolateral membrane. However, the targeting of this receptor may be more a function of its high homology to the adenine nucleotide-selective G_i-coupled $P2Y_{12}$ receptor than to its ligand. The significance of this unusual localization pattern is unclear, but one intriguing possibility may be that the distribution of P2Y receptors has evolved to compliment the preferential release of adenine nucleotides at the basolateral membrane and uridine nucleotides at the apical membrane. However, it is well documented that both ATP and UTP are released from the apical surface of epithelial cells in response to mechanical stimulation and hypotonic challenge (Homolya et al., 2000; Lazarowski et al., 1997a). In addition, Lazarowski and Harden have demonstrated a general release of UTP from primary epithelial cells (Lazarowski and Harden, 1999), although the relative amounts released from the two membrane surfaces is unknown and difficult to measure due to the complex nature of nucleotide metabolism and conversion that occurs within the interstitial space. Thus, the significance, if any, of this differential targeting of P2Y receptors remains unclear.

An important question in these studies was whether our targeting data with P2Y receptors in MDCK cells could be extrapolated to epithelial cells from other tissues. For example, it has been shown that the transferrin receptor, which is expressed on the basolateral surface in MDCK(II) cells, is expressed at the apical surface in porcine kidney LLC-PK1 cells (Folsch et al., 1999). This difference in targeting was subsequently attributed to the absence of a protein (the μ1B subunit of the adaptor protein AP1) in LLC-PK1 cells

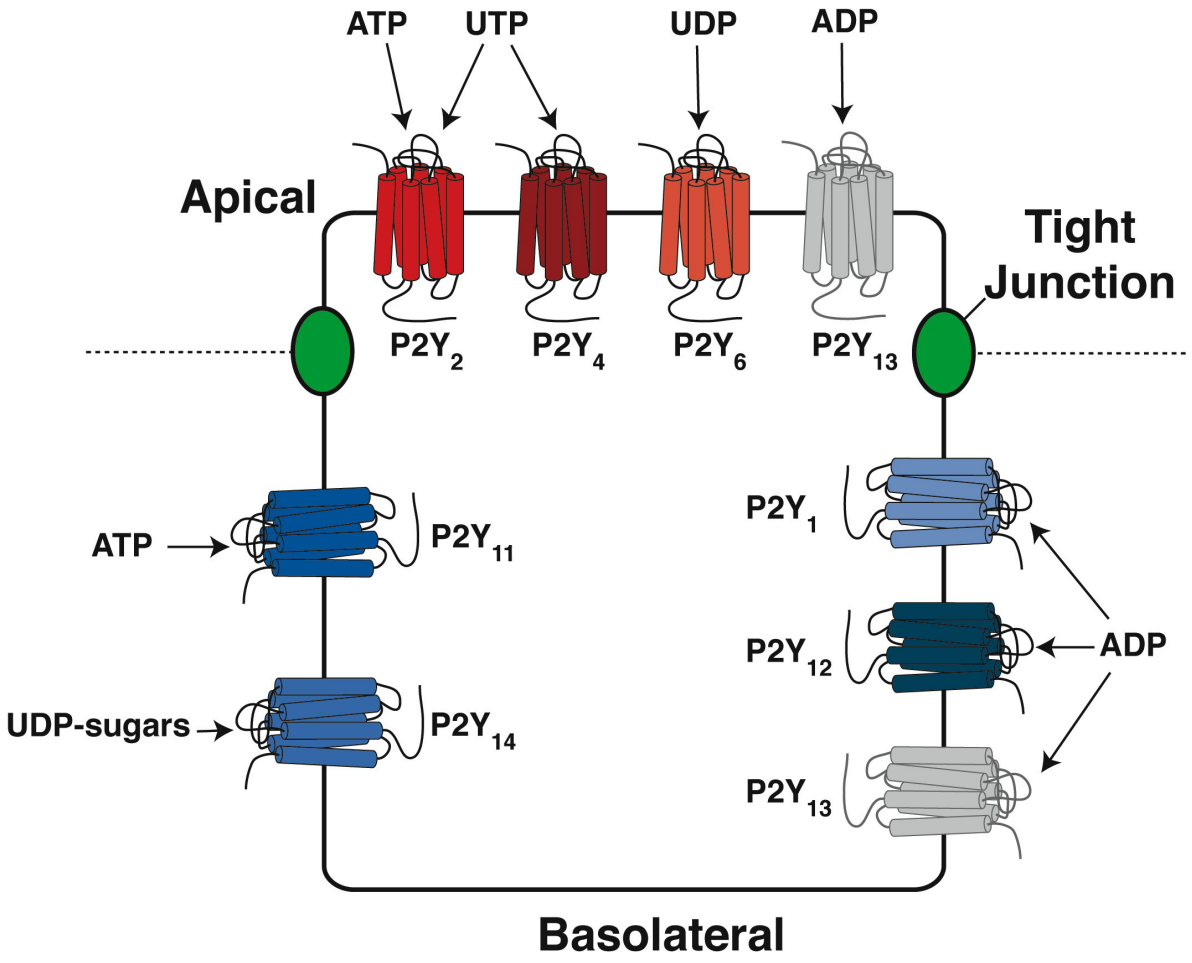


Figure 9. The P2Y receptor family and their polarized distribution in MDCK(II) epithelial cells.

The family of P2Y receptors and their cognate agonists are shown with their localization in polarized MDCK(II) cells. Receptors expressed at the apical surface ($P2Y_2$, $P2Y_4$, and $P2Y_6$) are shaded red, while those expressed at the basolateral surface ($P2Y_1$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{14}$) are shaded blue, and the unsorted receptor ($P2Y_{13}$) is gray. The dashed line demarcates the division between the apical and basolateral membrane domains. Receptors that are adenine nucleotide-selective are expressed at the basolateral surface, whereas receptors that are activated by uridine nucleotides are expressed at the apical surface.

(Folsch et al., 1999). To address this concern, we examined the targeting profiles of the Gq-coupled $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors in two other epithelial cell lines: 16HBE14o-, which is derived from bronchial epithelial cells transformed with the SV40 virus (Cozens et al., 1994), and CaCo-2, which is derived from a colonic adenocarcinoma (Fogh et al., 1977). Our data demonstrated that the polarized expression of the Gq-coupled P2Y receptor subtypes does not depend on the type of epithelial cell in which they are expressed (Fig. 7).

The only receptor that deviated somewhat from its targeting profile obtained in MDCK(II) cells was the $P2Y_2$ receptor, which in addition to its primarily apical localization was also expressed at lower levels along the lateral membranes of 16HBE14o- cells. Interestingly, this low level of lateral staining was also observed in another human epithelial cell line derived from lung, BEAS-2B (Reddel et al., 1988) (data not shown), but not in CaCo-2 cells (Fig. 7), suggesting that the small amount of lateral staining of the $P2Y_2$ receptor may be a property of airway cells in particular. Consistent with this observation, Boucher and co-workers (Homolya et al., 2000; Paradiso et al., 2001) demonstrated that UTP promoted intracellular Ca^{2+} mobilization when added to the basolateral surface of nasal epithelium derived from wild type mice. These responses were not observed in nasal epithelium derived from $P2Y_2$ receptor (-/-) mice, demonstrating that the responses are due to activation of basolateral $P2Y_2$ receptors. The physiological relevance of this observation is not clear, but our results suggest that the mechanisms utilized by epithelial cells to target the $P2Y_2$ receptor to the apical membrane are not as stringent in epithelial cells from lung compared to those from other tissues.

Although the polarized targeting of the Gq-coupled P2Y receptors is consistent with the majority of results based on functional activity (Dubyak, 2003; Leipziger, 2003; McAlroy et al., 2000; Zambon et al., 2001), our results conflict with several reports on the polarized expression of P2Y receptors in epithelial cells. For example, Sage and Marcus (Sage and Marcus, 2002) suggested a basolateral localization for $P2Y_2$ in vestibular dark epithelia based on immunostaining with a commercial antibody. However, results based on commercial $P2Y_2$ receptor antibody staining alone should be viewed with caution, since these antibodies exhibit questionable specificity for $P2Y_2$ receptors. A study by Nathanson and colleagues (Dranoff et al., 2001) utilized indirect pharmacological assays to suggest polarization of $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors at the apical membrane of rat bile duct epithelia. While demonstrating that these cells express multiple P2Y receptors, it is difficult to determine unequivocally which P2Y receptor subtypes are present at the apical membrane due to the complexities of tissues with unknown metabolizing and interconverting enzyme activities (Joseph et al., 2004; Lazarowski et al., 2003). Thus, without better reagents, including subtype-selective agonists and antagonists and antibodies with rigorously demonstrated receptor specificity, it is extremely difficult to show polarized targeting of P2Y receptors in complex tissues.

In conclusion, we have utilized three different approaches, including the direct method of confocal microscopy, to show the highly polarized expression pattern of the entire family of P2Y receptors. These data are for the most part consistent with previous reports and extend our knowledge of the localization of P2Y receptors in epithelial cells. Because the polarization of cell-surface proteins to either the apical or basolateral membrane of epithelial cells is achieved by the presence of targeting signals within the primary protein sequence, our

data suggest that seven of the eight hP2Y receptors contain targeting signals that direct their expression to one of the two membrane surfaces of MDCK cells. Studies to identify these targeting signals in P2Y receptors and to understand how these signals function are described in the next three chapters.

CHAPTER III: Localization of the targeting signals in $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{14}$ receptors

1. Introduction

Although P2Y receptors regulate multiple physiological processes in a variety of cells and tissues, one of their main roles is the regulation of ion transport and stress responses in epithelial cells (Insel et al., 2001). Epithelial cells line organs and cavities and provide an interstitial barrier between the internal environment of the body and the external world that surround all organisms. In addition, epithelia mediate numerous physiological functions at the interstitial interface, including regulated transport of ions, fluids, nutrients, and proteins, host defense, signal transduction, cell-cell interactions and cell-matrix adhesion (Brown, 2000). The structural and functional polarization of epithelia allows these cells to create a barrier and to carry out multiple physiological processes. The epithelial plasma membrane is divided into two distinct domains: the apical surface, which faces the lumenal space of an organ or external environment, and the basolateral surface, which is in contact with underlying cells and tissues. Each of these membrane domains has its own set of unique proteins and lipids. These membrane domains are separated by a protein-barrier complex known as the tight-junction, which forms a water- and ion-tight barrier between the two sides of the epithelial monolayer. The tight-junction is a complex of proteins anchored between the apical and basolateral domains by the cell's cytoskeleton and is a required component for epithelial function (Yeaman et al., 1999).

A large array of membrane-bound proteins unique to either the apical or basolateral membrane domains confers functional polarity to epithelial cells and allows processes specialized to the lumenal or internal space to occur. Furthermore, there is a high level of turnover for these plasma membrane (PM) bound proteins within epithelia, therefore requiring a high fidelity protein delivery system to maintain functional polarity (Brown and Breton, 2000). It has long been recognized that specific sorting information is located within the primary sequence of both transmembrane and cytosolic proteins that determines their locations in polarized cells, although the mechanisms by which they work has remained elusive. The elucidation of these sorting/targeting signals has been the focus of numerous laboratories (Brown and Breton, 2000; Folsch et al., 1999; Mostov et al., 2003).

Until recently, distinct sorting signals in G protein-coupled receptors (GPCRs) had not been identified. Much of the early work on identifying trafficking itineraries and targeting signals in GPCRs was carried out by Limbird and coworkers with α2-adrenergic receptors (Keefer and Limbird, 1993; Wozniak et al., 1997; Wozniak and Limbird, 1996; Wozniak and Limbird, 1998). These studies showed that three subtypes of α2-adrenergic receptors are delivered to the basolateral membrane by distinct targeting mechanisms that utilized multiple, non-contiguous targeting signals. In contrast to these results, more recent studies have identified linear targeting sequences in three different GPCRs. The cytoplasmic tail of rhodopsin was shown to act as a novel apical sorting signal in polarized MDCK cells (Chuang and Sung, 1998) through an interaction with the microtuble motor, dynein (Tai et al., 1999). In contrast, the C-terminal tail of the follicle stimulating (FSH) receptor contains a 14 amino acid basolateral targeting sequence (Beau et al., 1998) in which two amino acids, Tyr-684 and Leu-689, were found to be most important in determining basolateral targeting.

The M_3-muscarinic receptor contains a 21-amino acid sequence in its 3rd intracellular loop that functions as a basolateral targeting signal (Nadler et al., 2001).

These results suggest that linear targeting signals are likely to exist in GPCRs and are amendable to identification and characterization. However, given the small number of targeting sequences identified thus far for GPCRs, no consensus sequences have emerged. The paucity of studies on the targeting of GPCRs in general and more specifically for the P2Y receptor family led us to initiate a series of studies utilizing confocal microscopy and biotinylation assays to elucidate potential targeting signals within the seven P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{14}$) that were identified in the previous chapter as showing polarized expression.

2. Materials and methods

2.1 Approach/Rationale

In order to identify potential targeting signals in each of the polarized P2Y receptors, we utilized an approach wherein the localization of a series of chimeric and truncated constructs for each receptor subtype was examined by both confocal microscopy and biotinylation assays. This approach allowed us to identify the region of each receptor mediating either apical or basolateral targeting, as well as to test the ability of the newly identified signal to impart a specific trafficking itinerary onto receptors that were either unsorted and/or normally targeted to the opposite membrane. Once a region of the receptor harboring a targeting signal was identified it was deleted or disrupted to reveal if any additional targeting signals exist in the receptor. This search for additional signals was

conducted due to the fact that multiple targeting signals have been found in other receptor proteins, such as the follicle-stimulating hormone (FSH) receptor (Beau et al., 1998).

2.2 Construction of HA-tagged P2Y receptor chimeras and truncations

Two general methods were used to generate the HA-tagged P2Y receptor chimeras and truncation constructs utilized in this study. To construct chimeras, overlap extension PCR (Ho et al., 1989) using *Pfu* polymerase (Stratagene, La Jolla, CA) was employed. The outside primers contained an EcoRI restriction site at the 5'-end and a XhoI site at the 3'-end of the coding sequence. The full-length PCR products after the second round of amplification were digested with EcoRI and XhoI and ligated into similarly digested pLXSN retroviral expression vectors. To construct C-tail truncation mutants, PCR amplification was performed with 3'-primers containing a stop codon at the appropriate position and a XhoI site to facilitate cloning. In addition, each of the cloned receptors harbored an HA epitope tag (YPYDVPDY) following the initiating methionine residue. Previous studies have shown that the presence of an HA epitope at the N-terminus of a P2Y receptor has no effect of its function (Sromek and Harden, 1998).

2.3 Cell culture and expression of receptor constructs

Madin-Darby canine kidney type II cells (MDCK(II); ATCC, Rockville, Maryland) were subcultured in DMEM/F12 (1:1) medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS; Hyclone, Gaithersburg, MD) and 1X pen/strep in a humidified incubator at 37^{o}C with 5% CO_2 and 95% air.

Recombinant retroviral particles were produced by calcium phosphate-mediated transfection of PA317 cells with pLXSN vectors containing HA-tagged hP2Y receptor constructs as previously described (Comstock et al., 1997) and used to infect the cell line listed above. Geneticin-resistant cells were selected after 7-10 days with 1 mg/ml G418 and maintained in medium containing 0.4 mg/ml G418.

2.4 Confocal Microscopy

This assay was performed as described in Chapter II section 2.3 under the same heading.

2.5 Quantification of cell surface HA-tagged P2Y receptor constructs

This assay was performed as described in Chapter II section 2.4 under the same heading.

3. Results

3.1 Localization of the $P2Y_1$ and $P2Y_2$ receptor targeting signals

In order to determine the location of the basolateral targeting signal in the $P2Y_1$ receptor, we constructed a series of chimeras between the basolaterally targeted $P2Y_1$ receptor and the apically targeted $P2Y_2$ receptor and examined their steady-state localization in MDCK(II) cells by confocal microscopy. Of the chimeras tested, those in which only the C-terminal tails were swapped were most revealing. As shown in Figure 10, when the C-terminal tail of the $P2Y_1$ receptor was replaced with the C-terminal tail of the $P2Y_2$ receptor,

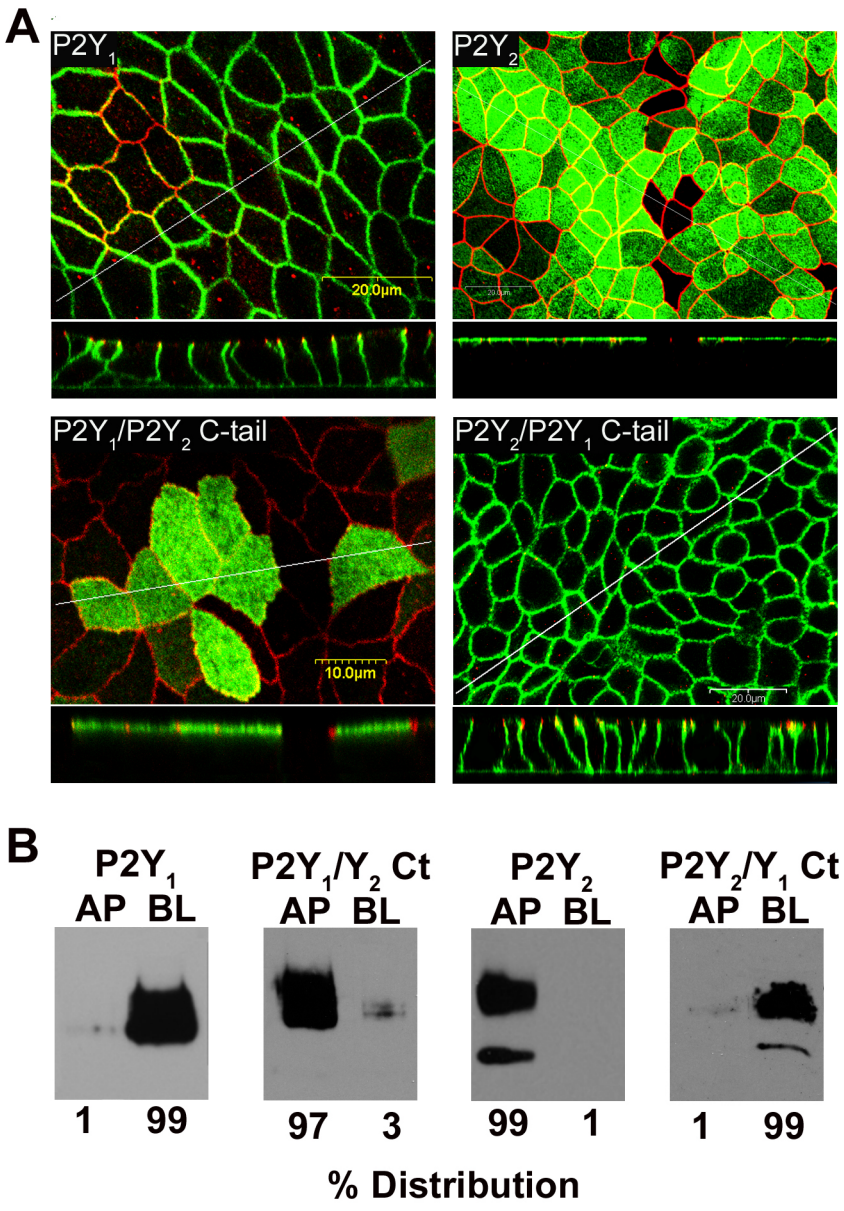


Figure 10. Localization of a targeting signal(s) in the $P2Y_1$ and $P2Y_2$ receptors.

A) The C-terminal tails of $P2Y_1$ and $P2Y_2$ receptors were swapped to investigate the possibility that this region of the protein contained basolateral and apical targeting signals, respectively. Confocal microscopy was utilized to determine the steady-state localization of these chimeras in polarized MDCK cells. Exchanging the C-tails of these two receptors switched their polarized localization, suggesting that the $P2Y_1$ C-tail contains a basolateral targeting signal and the $P2Y_2$ C-tail contains an apical targeting signal. Additional experiments (shown below) were carried out to confirm the possibility that these C-tails contain targeting signals. **B)** Cell surface biotinylation assays were carried out as described in Section 2.4 of Chapter II. The numbers below each lane represent the average percent distribution of the indicated receptor at each membrane domain (n=3).

the chimeric receptor was redirected to the apical membrane of MDCK(II) cells. Conversely, the $P2Y_2$ receptor containing the C-terminal tail of the $P2Y_1$ receptor was redirected to the basolateral membrane.

At first glance, the results of these experiments appeared to indicate that the C-terminal tail of the $P2Y_1$ receptor contains a basolateral targeting signal, while the C-terminal tail of the $P2Y_2$ receptor harbors an apical targeting signal. However, in order to further characterize the targeting signals in these proteins, we examined the localization of $P2Y_1$ and $P2Y_2$ receptors missing their C-terminal tails, as well as the normally unsorted B_2-bradykinin receptor in which its C-terminal tail was replaced by the C-terminal tail from either the $P2Y_1$ or $P2Y_2$ receptor. All four receptor constructs were expressed in MDCK(II) epithelial cells and analyzed by confocal microscopy and biotinylation assays (Fig. 11). The truncated $P2Y_2$ receptor ($P2Y_2$-ΔCT) was expressed at the apical membrane, but unexpectedly, the truncated $P2Y_1$ receptor ($P2Y_1$-ΔCT) was also expressed exclusively at the apical membrane. Moreover, confocal microscopy revealed that the $BK_2/P2Y_1$ C-tail receptor was expressed entirely at the basolateral surface, while the BK_2-$P2Y_2$ C-tail receptor was unsorted. These results are consistent with the idea that the $P2Y_1$ receptor contains two signals: an apical signal located somewhere between the N-terminus and TM7, and a basolateral signal in the C-terminal tail that is dominant over the apical signal. Thus, redirection of the $P2Y_1$ receptor containing the $P2Y_2$ C-terminal tail to the apical membrane was not due to the addition of the $P2Y_2$ C-terminal tail but to uncovering the secondary apical signal upon removal of the dominant basolateral signal in the C-terminal tail. In addition, these results demonstrate that the apical targeting signal in the $P2Y_2$ receptor is located between its N-terminus and TM7 (the main body of the protein).

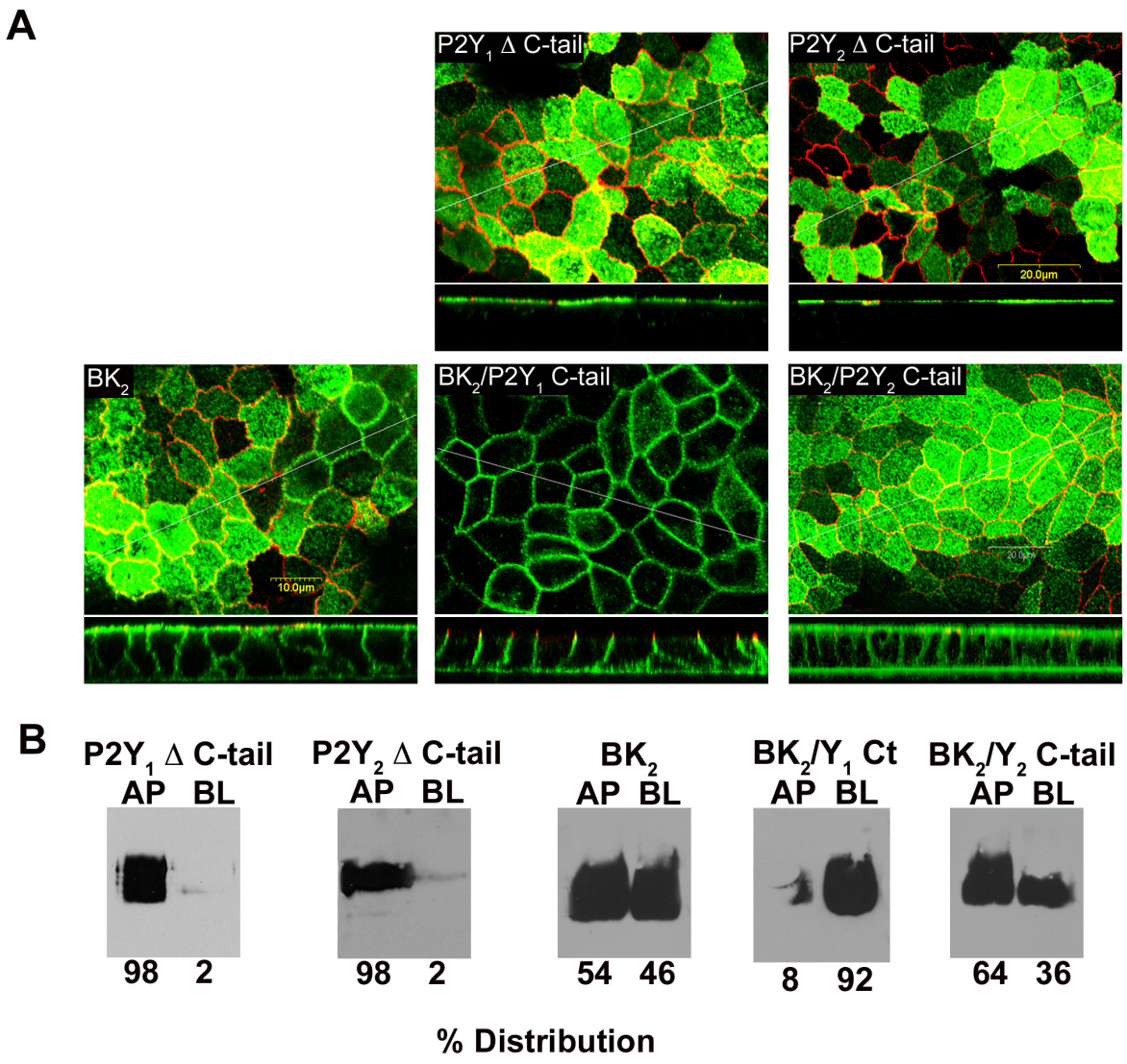


Figure 11. Additional localization experiments elucidating the targeting signals in the $P2Y_1$ and $P2Y_2$ receptors.

A) To determine the location of a targeting signal(s) in the $P2Y_1$ receptor we deleted the C-tail of the receptor ($P2Y_1$ Δ 334) as well as replaced the C-tail of the normally unsorted bradykinin-2 receptor (BK_2) with the C-tail of the $P2Y_1$ receptor (BK_2/Y_1 CT), expressed these receptors in MDCK(II) cells, and determined their steady-state distribution in polarized monolayers by confocal microscopy. The same approach was taken for the localization of targeting signals in the $P2Y_2$ receptor. **B)** Cell surface biotinylation assays were carried out as described in Section 2.4 of Chapter II. The numbers below each lane represent the average percent distribution of the indicated receptor at each membrane domain (n=3).

3.2 Identification of the $P2Y_4$ Receptor Apical Targeting signal

Based on previous experiments with the $P2Y_1$ receptor, which demonstrated a targeting signal present in the C-tail, we replaced the endogenous C-tail of the unsorted BK_2 receptor with the C-tail of the $P2Y_4$ receptor, resulting in a $BK_2/P2Y_4$ C-tail chimera. In addition, we truncated the $P2Y_4$ receptor just after TM7 thereby removing its C-tail ($P2Y_4$ Δ C-tail). Both of these receptor constructs were expressed in MDCK(II) cells and their steady-state localization was determined by confocal microscopy and surface biotinylation. Whereas the BK_2 receptor is normally unsorted, the $BK_2/P2Y_4$ C-tail chimera was redirected to the apical membrane. Likewise, the $P2Y_4$ receptor is expressed exclusively at the apical membrane, while the $P2Y_4$ Δ C-tail receptor was unsorted (i.e. it localized equally to both the apical and basolateral membranes)(Fig. 12). Taken together, these results demonstrate that the $P2Y_4$ receptor contains an apical targeting signal in its C-tail.

3.3 Localization of the apical targeting signals in the $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors (in collaboration with Aidong Qi)

Our previous studies demonstrated that three out of eight P2Y receptor subtypes ($P2Y_2$, $P2Y_4$, and $P2Y_6$) are targeted to the apical membrane of MDCK(II), 16HBE14o- and CaCo-2 epithelial cells (Wolff et al., 2005). In experiments described above, the targeting signal responsible for apical localization of the $P2Y_4$ receptor is located in its C-tail, while the apical signal for the $P2Y_2$ receptor is located between the N-terminus and TM7 (the main body) of the protein. Because these two apically-targeted receptor subtypes share high sequence identity (52%) and have distinctly different locations of their targeting signals, we

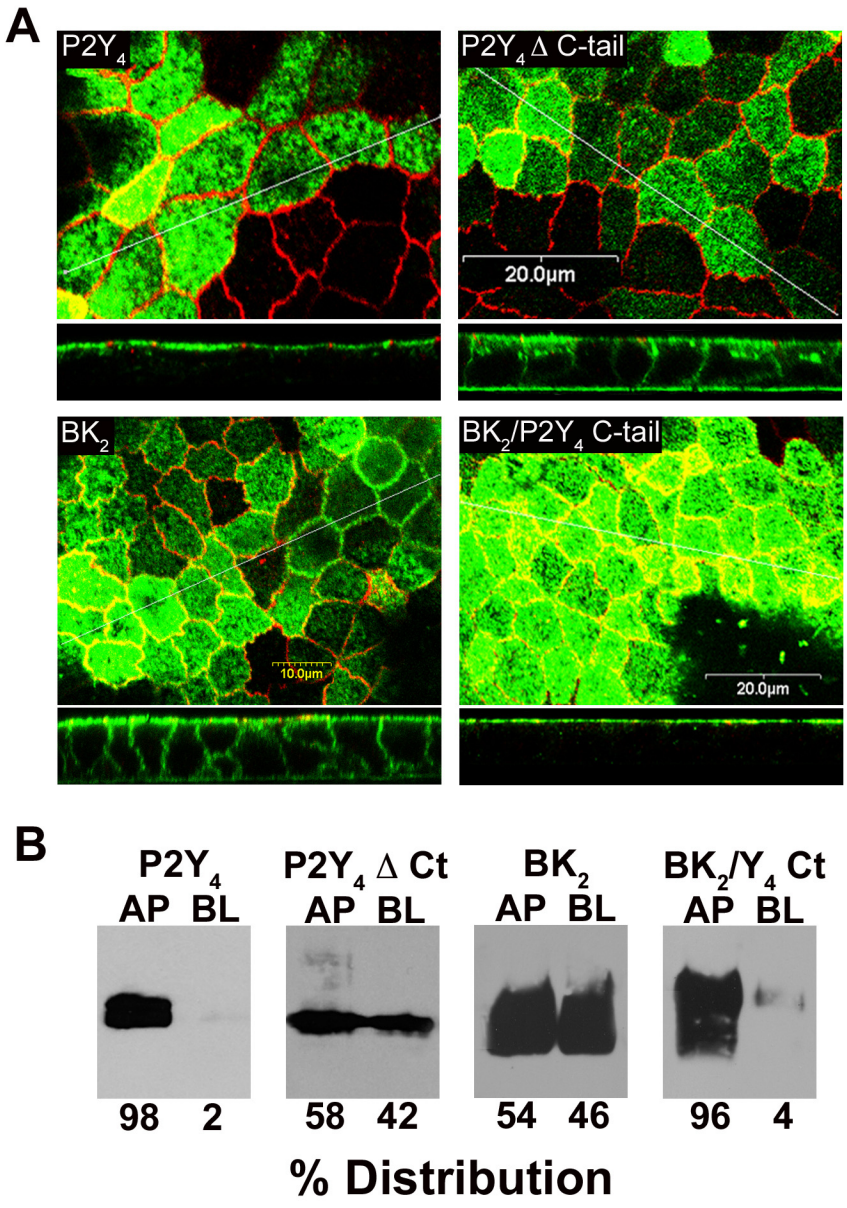


Figure 12. Localization of the $P2Y_4$ receptor apical targeting signal.

A) In order to localize the apical targeting signal of the $P2Y_4$ receptor two types of constructs were generated: 1) a truncated form of the $P2Y_4$ receptor wherein the C-tail is removed and 2) a chimera of the unsorted human Bradykinin-2 (BK_2) receptor in which its C-tail was replaced by the $P2Y_4$ C-tail. These receptor constructs were expressed in MDCK(II) cells for confocal analysis. **B)** Cell surface biotinylation assays were carried out as described in Section 2.4 of Chapter II. The numbers below each lane represent the average percent distribution of the indicated receptor at each membrane domain (n=3).

were able to localize the targeting signal in the $P2Y_2$ receptor. We constructed a series of chimeric receptors by progressively substituting regions of the $P2Y_2$ receptor with the corresponding regions of the $P2Y_4$ receptor lacking its targeting signal, and confocal analysis of these chimeras revealed that the targeting signal is located in the first extracellular loop (EL1) of the $P2Y_2$ receptor (data not shown). Subsequent mutational analysis of EL1 demonstrated that four amino acids (R^{95}, G^{96}, D^{97}, and L^{108}) play a major role in apical targeting of the $P2Y_2$ receptor (Qi et al., 2005).

In experiments described in section 3.1, we localized a secondary apical targeting signal for the $P2Y_1$ receptor to its main body. In addition, our previous study on P2Y receptor localization in epithelial cells demonstrated that the $P2Y_6$ receptor subtype has an apical targeting profile (Wolff et al., 2005). In light of these data, we aligned the primary sequences of EL1 in these two receptor subtypes with the corresponding sequence in the $P2Y_2$ receptor to uncover any conserved amino acid motifs that potentially may be involved in receptor targeting. Indeed, this alignment showed that R^{95}, G^{96}, D^{97}, and L^{108} of the $P2Y_2$ receptor are largely conserved in the $P2Y_6$ ($Q^{88}G^{89}D^{90}$ and L^{101}) and $P2Y_1$ ($N^{113}K^{114}T^{115}D^{116}$ and L^{126}) receptors (Fig. 13). Mutagenesis of each of these conserved amino acids (with the exception of D^{90} in the $P2Y_6$ receptor, which resulted in the intracellular accumulation of the mutant receptor) in both the $P2Y_6$ and $P2Y_1$ receptors caused a dramatic impairment in apical targeting (data not shown). These data reveal that EL1 of $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors contains a conserved apical targeting signal.

Figure 13. Sequence Alignment of $P2Y_2$, $P2Y_6$, and $P2Y_1$ receptor EL1.

The primary sequence of the first extracellular loop (EL1) for the $P2Y_6$ and $P2Y_1$ receptors are aligned with the region (EL1) of the $P2Y_2$ receptor that contains its apical targeting signal with key targeting residues highlighted in red. This alignment was performed in order to uncover any conserved motifs that may be potentially involved in the apical targeting of the $P2Y_6$ and $P2Y_1$ receptors. This alignment shows that key amino acids (red) involved in the targeting of the $P2Y_2$ receptor are conserved in both the $P2Y_6$ and $P2Y_1$ receptors, which are highlighted in blue. Based upon this analysis, mutagenesis studies were carried out on the amino acids identified as potential mediators of targeting for the $P2Y_6$ and $P2Y_1$ receptors.

3.4 Localization of the basolateral targeting signals in the $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{14}$ receptors

$P2Y_{11}$, $P2Y_{12}$ and $P2Y_{14}$ receptors are localized at steady-state to the basolateral membrane of MDCK(II) cells (Wolff et al., 2005). Because the basolateral and apical targeting signals of $P2Y_1$ and $P2Y_4$ receptors, respectively, are located in their C-tails, we hypothesized that the basolateral sorting signals for $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{14}$ receptors also might be located in their C-tails. In order to test this hypothesis, we replaced the C-tails of the unsorted BK_2 receptor or the apically-targeted $P2Y_2$ receptor with the C-tails from the $P2Y_{11}$, $P2Y_{12}$ or $P2Y_{14}$ receptor. These chimeras were expressed in MDCK(II) cells and the steady-state localization of each receptor chimera was determined by confocal microscopy. Confocal analysis demonstrated that all three receptor chimeras were redirected to the basolateral membrane of MDCK(II) cells, suggesting that the C-tails of these receptors contain a sorting signal that is both necessary and sufficient to direct a protein to the basolateral membrane (Fig. 14).

We also examined the main body of the three receptors for the presence of secondary sorting signals. The C-tails of the $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{14}$ receptors were removed by putting in a stop codon just past TM7 and expressing the truncated receptors in MDCK(II) cells. Unfortunately, expression of the truncated $P2Y_{11}$ and $P2Y_{14}$ receptors resulted in unstable receptors that did not reside on the plasma membrane. In contrast, confocal microscopy of the truncated $P2Y_{12}$ receptor showed that the receptor was localized to the basolateral membrane, suggesting the existence of a redundant BL sorting signal located between the N-terminus and TM7 (Fig. 15).

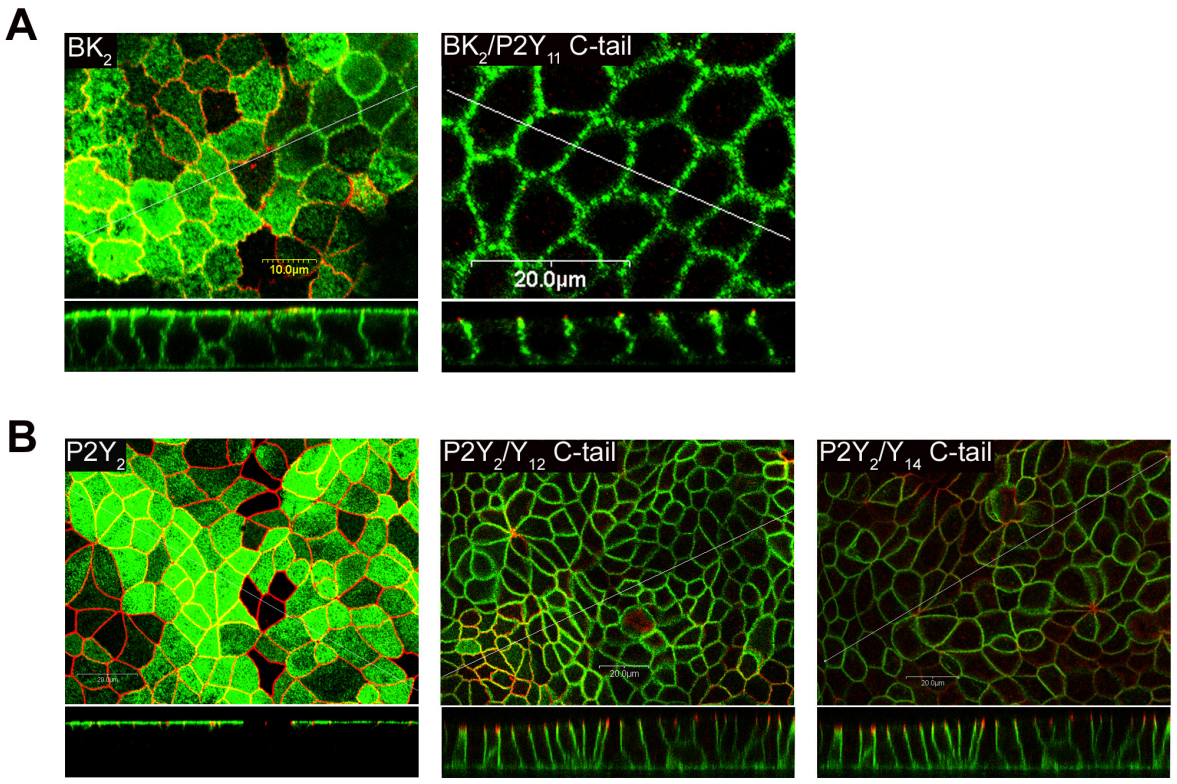


Figure 14. Localization of targeting signals for $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{14}$ receptors.

Targeting signals for the $P2Y_1$ and $P2Y_4$ receptors are located in the C-tail, therefore, we hypothesized that this intracellular region of the receptor may contain sorting information for the $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{14}$ receptors as well. This hypothesis was investigated by constructing three chimeras in which we replaced the C-tail of the normally unsorted BK_2 receptor with the $P2Y_{11}$ receptor C-tail **(A)** and replacing the C-tail of the normally apical $P2Y_2$ receptor with the $P2Y_{12}$ or $P2Y_{14}$ receptor C-tails **(B)**. These chimeric receptors were expressed in MDCK(II) epithelial cells and examined for changes in steady-state localization by confocal microscopy.

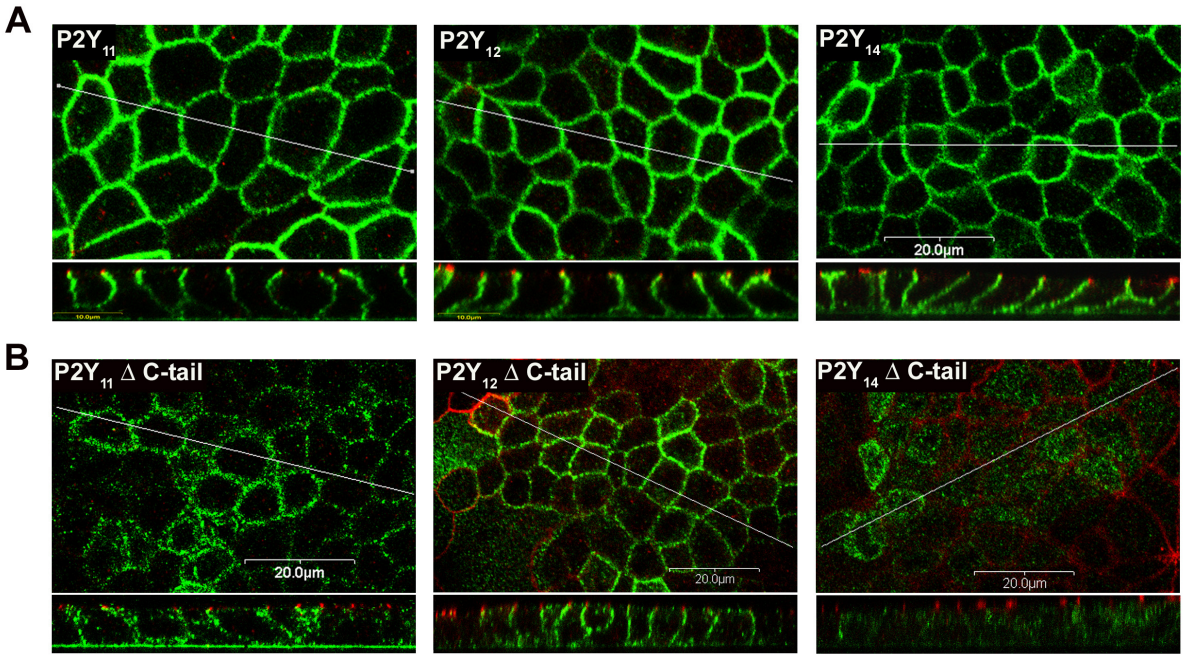


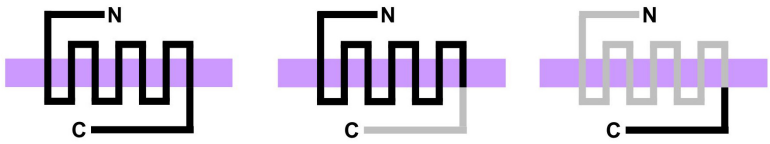
Figure 15. Identification of secondary targeting signals for the $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{14}$ receptors.

Secondary targeting signals have been shown to exist in GPCR's including the $P2Y_1$ receptor. Therefore, we examined the $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{14}$ receptors for such signals in a truncation experiment. All three receptors are normally localized to the basolateral surface (**A**), which is due to the existence of targeting signals in their C-tails. In order to uncover any secondary signals, the locus of the primary signals (the C-tail) was removed by inserting a stop codon just after TM7 thereby creating a truncated receptor. These truncated receptors ($P2Y_{11}$ Δ C-tail, $P2Y_{12}$ Δ C-tail and $P2Y_{14}$ Δ C-tail) were expressed in MDCK(II) epithelial cells and examined with confocal microscopy for steady-state localization (**B**).

4. Discussion

In this series of experiments we localized both the primary and secondary targeting signals in all of the polarized P2Y receptors, which include the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{14}$ subtypes. The majority of these receptors ($P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and $P2Y_{14}$) contain a single apical- or basolateral-targeting signal that constitutes the primary sorting information for the receptor. In contrast, $P2Y_1$ and $P2Y_{12}$ receptors contain more than one signal. In the case of the $P2Y_1$ receptor, the C-tail harbors a primary basolateral-targeting signal that overrides a secondary apical signal located in the first extracellular loop. The $P2Y_{12}$ receptor was unusual in that it contains two basolateral targeting signals: one located in the C-tail and another located somewhere in the main body. Furthermore, we described the identification of the apical targeting signal located in the first extracellular loop of the $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors. The number and location of the targeting signals in the P2YR family is summarized in Table 3.

The experiments described in this chapter were conducted because they were the next logical step in the progressive characterization of P2Y receptor targeting properties. In the previous chapter (Chapter II), we demonstrated the polarized distribution of seven of the eight human P2Y receptors in a variety of epithelial cell lines. Based upon the well-established principles of protein targeting described in the literature (Brown and Breton, 2000; Mostov et al., 2003; Mostov et al., 2000), we hypothesized that sorting signals contained in the primary sequence of P2Y receptors are responsible for the targeting of these proteins to either the apical or basolateral membrane of epithelial cells. As shown in this chapter a series of experimental results does indeed confirm this hypothesis.



P2Y subtype	Full Receptor	Main Body	C-tail
$P2Y_1$	BL	AP	BL
$P2Y_2$	AP	AP	No signal
$P2Y_4$	AP	No Signal	AP
$P2Y_6$	AP	AP	No Signal
$P2Y_{11}$	BL	No Signal	BL
$P2Y_{12}$	BL	BL	BL
$P2Y_{14}$	BL	?	BL

Table 3. Location of sorting signals in P2Y receptors.
All of the delimited targeting signals in the P2Y receptor family are shown along with their locations within each receptor. Please note that receptors can harbor multiple sorting signals. AP, apical; BL, basolateral; ?, unknown.

Once this hypothesis was confirmed the next logical step in our investigation was to fully characterize each of the P2Y receptor targeting signals. In approaching these experiments, we took advantage of the fact that basolateral targeting signals are typically short amino-acid motifs located within the cytoplasmic domain of the targeted protein. The best-characterized signal is the 4-amino acid tyrosine-based motif (YxxF) that not only is sufficient to direct targeting to the basolateral membrane, but has been shown to interact with adaptor proteins (AP) to mediate endocytosis (Mostov et al., 1999). The duality of this signal suggests an alternative pathway for protein trafficking exists wherein proteins move from the TGN to a common/sorting endosome first and then are delivered to a specific membrane compartment. Another well-characterized sorting signal is the di-leucine motif, which has been shown to deliver a number of proteins to the basolateral membrane (Rodriguez-Boulan et al., 2005). This sequence is also thought to interact with adaptor proteins during transport to the basolateral surface. Finally, the importance of secondary protein structure in basolateral targeting signals has been suggested. For example, the 17-amino acid sorting signal found in the polymeric immunoglobulin receptor (pIgR) has been shown to adopt a beta-turn structure that is critical for its polarized targeting (Reich et al., 1996).

In contrast to BL targeting signals, identification of sorting signals for apically-bound proteins have proven to be more elusive. Such signals have been found in the extracellular, transmembrane and intracellular domain of proteins. Proteins containing a covalently linked glycerophosphatidyl-inositol modification are sorted to the apical membrane of many (but not all) polarized epithelial cell types (Brown et al., 1989; Lisanti et al., 1989). This lipid modification is thought to associate with membrane structures called rafts, which are composed of clustered glycosphingolipids, cholesterol, and certain other proteins (Mostov et

al., 2000). In addition, both N- and O-linked oligosaccharides have been suggested to act as apical sorting signals (Scheiffele et al., 1995; Yeaman et al., 1997), but the role of glycosylation as a sorting signal is unclear. Furthermore, we have identified and described the first protein-based apical signal, which is located in the 1st extracellular loop of the $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors. Apical targeting sequences also are located within transmembrane (TM) domains. For example, the fourth TM domain of the gastric H^+-ATPase directs apical expression (Dunbar et al., 2000). Finally, a role of C-terminal PDZ-binding domains in localization of proteins to the apical surface is emerging. For example, the PDZ-binding domain of the CFTR is critical for its apical localization and its interaction with the PDZ domain-containing protein, EBP50 (Moyer et al., 1999; Moyer et al., 2000).

In the elucidation of targeting signals investigators typically utilize a mutagenesis approach to identify key amino acids involved in protein targeting. Some of the key studies described above have successfully utilized this approach and have been essential in the illumination of common motifs (such as they are) to both basolateral and apical targeting signals. In order to extend our knowledge on P2Y receptor targeting signals and to make similar contributions to the protein targeting knowledge base, we carried out similar experiments on the $P2Y_1$ and $P2Y_4$ receptors, which are described in the next two chapters (Chapters IV and V).

CHAPTER IV: The C-terminal tail of the $P2Y_1$ receptor contains a novel basolateral sorting signal: Importance of charged residues and lack of sequence specificity in signal function

1. Introduction

Molecular cloning and functional characterization has identified eight P2Y receptor subtypes ($P2Y_{1,2,4,6,11,12,13,14}$). $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors are members of the $P2Y_1$ receptor subfamily and are coupled to activation of phospholipase C, generation of inositol phosphates and mobilization of intracellular Ca^{2+} stores (Harden, 1998; Ralevic and Burnstock, 1998). In addition to coupling to activation of phospholipase C, the $hP2Y_{11}$ receptor also couples to Gs and activation of adenylyl cyclase (Communi et al., 1997; Qi et al., 2001a; Torres et al., 2002). In contrast, the three most recently identified P2Y receptors, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$, are members of the $P2Y_{12}$ receptor subfamily and couple solely to Gi/o and inhibition of adenylyl cyclase (Chambers et al., 2000; Communi et al., 2001; Hollopeter et al., 2001; Zhang et al., 2002).

Although P2Y receptors regulate multiple physiological processes in a variety of cells and tissues, one of their main roles is the regulation of ion transport and stress response in epithelial cells (Bucheimer and Linden, 2004; Leipziger, 2003; Nishiyama et al., 2004; Schwiebert and Zsembery, 2003). Epithelial cells form an interstitial barrier between the external environment and internal cells and tissue, and thereby mediate numerous physiological functions at the interstitial interface, including regulated transport of ions,

fluids, nutrients, and proteins, host defense, signal transduction, cell-cell interactions and cell-matrix adhesion (Brown, 2000). The structural and functional polarization of epithelia allows these cells to create a water- and ion-tight barrier that provides exquisite regulation of ion and liquid fluxes across the epithelial monolayer. The plasma membranes of these cells are divided into two distinct domains, apical and basolateral, each with its own unique set of proteins and lipids. The apical membrane faces the lumen, whereas the basolateral membrane contacts other cells of the monolayer and the underlying cells and connective tissue.

Many of the membrane-bound proteins expressed in polarized epithelial cells are localized to either the apical or basolateral domain, which confers functional polarity to epithelial cells and allows processes specialized to the lumenal or basolateral spaces to occur. It has long been recognized that specific sorting information is located within the primary sequences of these proteins that determines their locations in polarized cells, although the mechanisms by which they work has remained elusive. Elucidation of these sorting/targeting signals has been the focus of numerous laboratories.

Recent work from our laboratory has demonstrated that seven of the eight P2Y receptors are expressed in a polarized manner in epithelial cells from kidney, lung, and colon (summarized in Chapter II) (Wolff et al., 2005). Of these seven receptors, four ($P2Y_1$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{14}$) are localized to the basolateral membrane. The conserved polarized targeting of the Gq-coupled receptors led us to investigate whether the polarized expression of the $P2Y_1$ receptor was due to the presence of a basolateral targeting signal. To begin to understand how receptors are localized to the basolateral membrane, we constructed a series of P2Y chimera and mutant receptors in order to delimit the targeting signal. Our experiments demonstrate that this sorting signal is a 25-amino acid cassette located in the C-terminus tail

(C-tail) of the receptor and is dependent on charged amino acids (both negative and positive) but not sequence specificity in order to function. Furthermore, the existence of a secondary apical targeting signal in the 1st extracellular loop of the $P2Y_1$ receptor was uncovered and is described in Chapter III. This is a first report of a basolateral-targeting signal that functions solely by containing a critical mass of charged amino acids located in close proximity to the plasma membrane.

2. Materials and Methods

2.1 Construction of HA-tagged $P2Y_1$ and $P2Y_2$ receptor chimeras, mutants and truncations

Three general methods were used to generate the HA-tagged P2Y receptor chimeras, truncations, and mutant constructs utilized in this study. To construct chimeras and simple mutants, overlap extension PCR (Ho et al., 1989) using *Pfu* polymerase (Stratagene, La Jolla, CA) was employed. The outside primers contained an EcoRI restriction site at the 5'-end and a XhoI site at the 3'-end of the coding sequence. The resulting PCR products were digested with EcoRI and XhoI and ligated into similarly digested pLXSN retroviral expression vectors. To construct truncation mutants, PCR amplification was performed with 3'-primers containing a stop codon at the appropriate position and a XhoI site to facilitate cloning.

To construct receptors containing multiple point mutations, long overlapping primers (~ 60 bases) encoding various mutations within the C-tail of the $P2Y_1$-364Z receptor were utilized. The sense primer contained a XhoI restriction site and the antisense primer contained a BamHI site, respectively, at the 5' end. The primers overlapped by approximately 18 bases in the middle of the target sequence. The primers were annealed,

filled-in with the Klenow fragment of DNA polymerase I (New England BioLabs, Beverly, MA), digested with XhoI and BamHI, and the small double-stranded fragment was ligated into a similarly digested pLXSN-HA-$P2Y_1$-339Z plasmid in which a XhoI site was created by silent mutation of codon-336 and a BamHI site was incorporated at the end of the coding sequence. Similar methods were used to make $P2Y_2$- and BK_2-$P2Y_1$ C-tail mutants, with the fusion sites at $P2Y_2$-R315 and BK_2-R313, respectively. In addition, each of the cloned receptors harbored an HA epitope tag (YPYDVPDY) following the initiating methionine residue. Previous studies have shown that the presence of an HA epitope at the N-terminus of a P2Y receptor has no effect of its function (Sromek and Harden, 1998).

2.2 Cell culture and expression of receptor constructs

Madin-Darby canine kidney type II cells (MDCK(II); ATCC, Rockville, Maryland) were subcultured in DMEM/F12 (1:1) medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS; Hyclone, Gaithersburg, MD) and 1X pen/strep in a humidified incubator at 37^{o}C with 5% CO_2 and 95% air. LLC-PK1 cells were subcultured in alpha medium supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1X pen/strep in a humidified incubator at 37^{o}C with 5% CO_2 and 95% air.

Recombinant retroviral particles were produced by calcium phosphate-mediated transfection of PA317 cells with pLXSN vectors containing HA-tagged hP2Y receptor constructs as previously described (Comstock et al., 1997) and used to infect the cell lines listed above. Geneticin-resistant cells were selected after 7-10 days with 1 mg/ml G418 and maintained in medium containing 0.4 mg/ml G418.

2.3 Confocal Microscopy

This assay was performed as described in Chapter II section 2.3 under the same heading.

2.4 Quantification of cell surface HA-tagged P2Y receptor constructs

This assay was performed as described in Chapter II section 2.4 under the same heading.

3. Results

3.1 Delimitation of the basolateral targeting signal in the $P2Y_1$ receptor

As shown in section 3.1 of Chapter III, the $P2Y_1$ receptor C-tail contains a basolateral targeting signal that is dominant over a secondary apical targeting signal located in its main body. In order to delimit the basolateral targeting signal of the $P2Y_1$ receptor, we constructed a series of C-tail truncations. These experiments took advantage of the cryptic apical signal in the main body of the receptor, which redirects the receptor to the apical membrane upon disruption of the dominant basolateral targeting signal. Given the role of PDZ-binding motifs in protein targeting (Altschuler et al., 2003), we first examined the localization of the $P2Y_1$-369Z receptor, which was missing the last four amino acids (DTSL) that fit a classic type I PDZ-binding motif (Fam et al., 2005). Confocal analysis of this receptor demonstrated that there was no change in basolateral targeting, eliminating the PDZ-binding motif as a targeting signal (Fig. 16). Deletion of the next five amino acids of the C-terminal tail ($P2Y_1$-

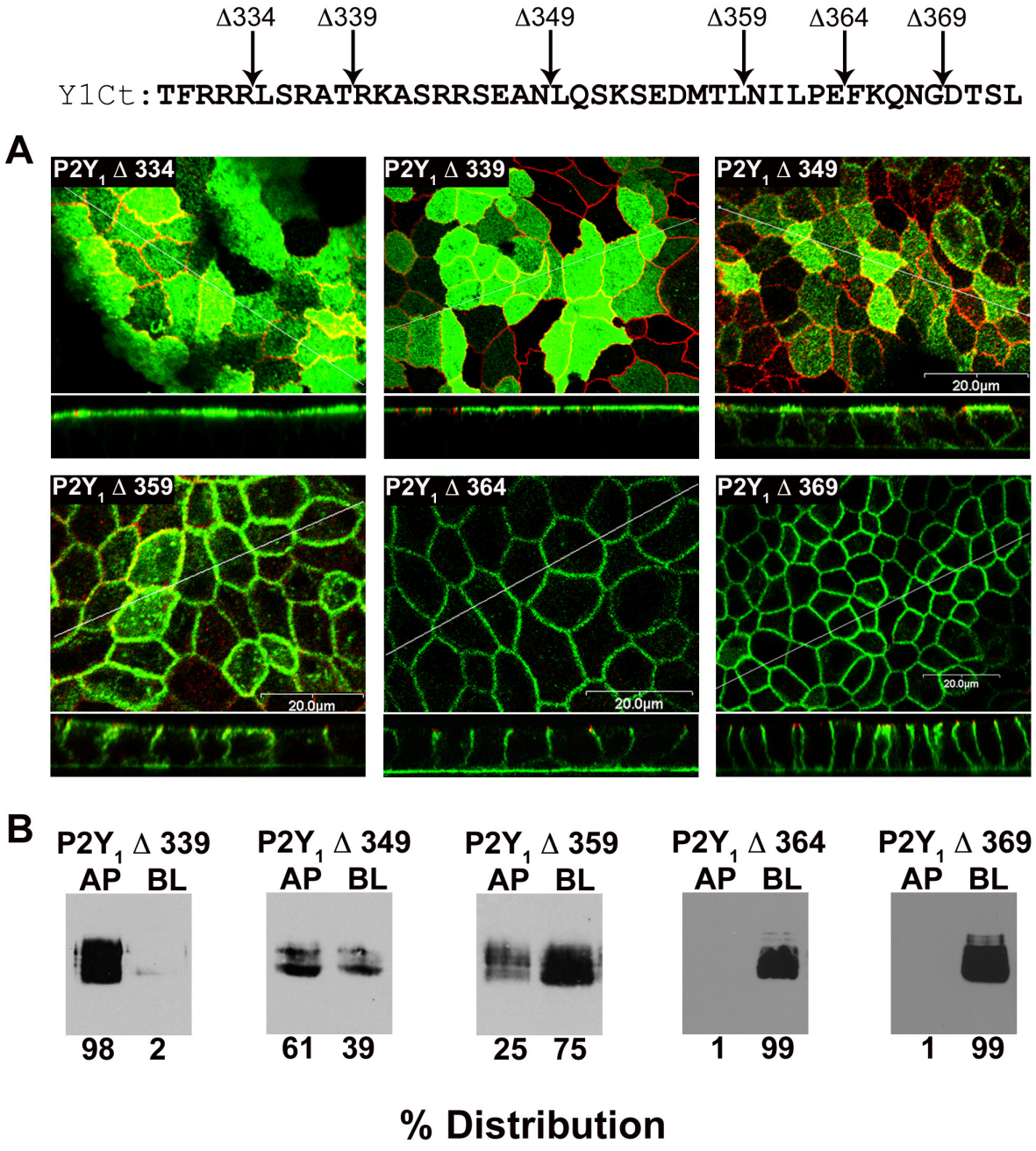


Figure 16. Localization of P2Y$_1$ Δ C-tail receptors.

A) A series of truncations in the C-tail of the P2Y$_1$ receptor was constructed and the resulting receptors were stably expressed in polarized MDCK cells. The localization of these truncated receptors was analyzed by confocal microscopy in order to delimit the basolateral targeting signal. The basolateral targeting signal is contained within a stretch of 25 amino acids between amino acids Thr^{339} and Glu^{364}. **B)** Cell surface biotinylation assays were carried out as described in Section 2.4 of Chapter II. The numbers below each lane represent the average percent distribution of the indicated receptor at each membrane domain (n=3).

364Z) also had no effect on basolateral targeting (Fig. 16). In contrast, further removal of 5, 15, or 25 amino acids ($P2Y_1$-359Z, $P2Y_1$-349Z and $P2Y_1$-339Z, respectively) of the C-terminal tail resulted in the gradual redistribution of the receptor from the basolateral to the apical surface. Truncation of 34 amino acids of the $P2Y_1$ receptor C-tail ($P2Y_1$-339Z) resulted in the complete redistribution of the receptor to the apical membrane. These data suggested that the basolateral-targeting signal (referred to hereafter as the basolateral-targeting cassette or BLC) in the $P2Y_1$ receptor is 25 amino acids in length and is located between Arg^{340} and Glu^{364} (Fig. 16). As above, biotinylation experiments were in complete agreement with the distributions determined by confocal microscopy (Fig. 16B).

To verify that no other amino acids were necessary for directing basolateral targeting, we fused the 25-amino-acid BLC to R^{315} of the $P2Y_2$ receptor ($P2Y_2$-315/BLC), which is just four amino acids beyond the predicted end of TM7. Whereas the truncated $P2Y_2$–315Z receptor was expressed at the apical membrane, the $P2Y_2$ receptor containing the $P2Y_1$ BLC was expressed exclusively at the basolateral membrane (Fig. 17). Thus, the 25-amino-acid BLC acts as an autonomous signal capable of directing different receptors to the basolateral membrane.We next examined the importance of the location of the BLC relative to TM7 in its ability to direct basolateral targeting. For these experiments, we fused the BLC at different points along the primary sequence of the C-terminal tail of the $P2Y_2$ receptor. As described above, fusion of the BLC to R^{315} of the $P2Y_2$ receptor targets the receptor completely to the basolateral membrane (Fig. 17). In contrast, the BLC began to lose its ability to direct basolateral targeting when fused following G^{337} of the $P2Y_2$ receptor (82% basolateral), which was 26 amino acids removed from TM7, and almost completely lost its ability to

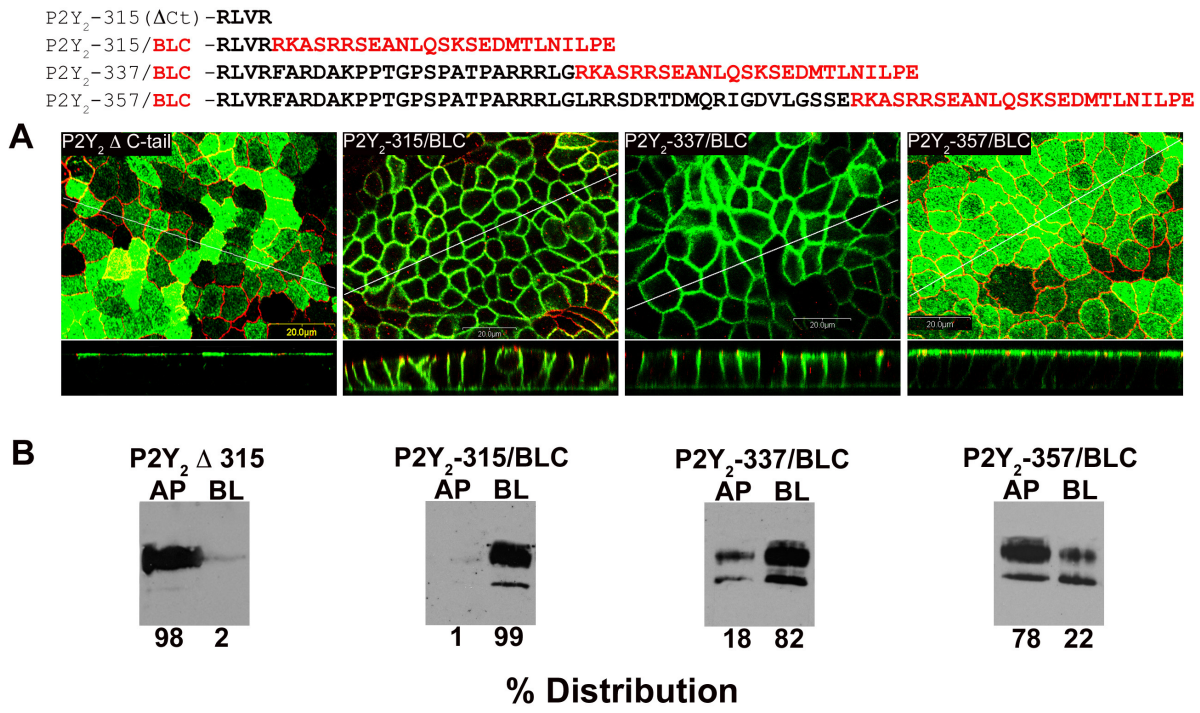


Figure 17. Distance from the membrane is important for the functioning of the basolateral cassette (BLC).

A) Previous experiments show that the replacement of the endogenous $P2Y_2$ C-tail with the BLC redirected this apically targeted receptor to the basolateral membrane. In order to test the effect of distance on BLC function, the BLC was placed further and further away from the plasma membrane surface along the $P2Y_2$ C-tail. These $P2Y_2$ C-tail/BLC chimeras were expressed in polarized MDCK(II) cells and examined by confocal microscopy and quantitative biotinylation assays in order to determine receptor localization. **B)** Cell surface biotinylation assays were carried out as described in Section 2.4 of Chapter II. The numbers below each lane represent the average percent distribution of the indicated receptor at each membrane domain (n=3).

direct basolateral targeting (22% basolateral) when fused 46 amino acids from TM7 (following E^{357}). $P2Y_2$ receptors truncated after G^{337} and E^{357} but lacking the $P2Y_1$ BLC were targeted to the apical membrane, demonstrating that the observed basolateral targeting was due to the action of the BLC (data not shown). These data suggest that the location of the BLC relative to TM7 is critical in its ability to confer basolateral targeting.

Based on these results, we also tested whether the BLC was capable of mediating membrane binding by examining its ability to confer basolateral targeting to a normally soluble, cytoplasmic protein such as green fluorescent protein (GFP). We expressed wild-type GFP and GFP containing the BLC sequence at its C-terminus (GFP/BLC) in MDCK(II) cells and analyzed the localization of these proteins by confocal microscopy. A diffuse distribution pattern throughout the cytoplasm was observed for both GFP and GFP/BLC (Fig. 18), demonstrating that the BLC does not promote membrane association and that it must be part of a membrane-bound protein in order to function.

3.2 Role of μ1B in basolateral targeting of the $P2Y_1$ receptor

Previous studies have demonstrated that the μ1B subunit of the adaptor protein complex AP1 plays an important role in the basolateral targeting of certain receptors such as the transferrin receptor (Folsch et al., 1999). The porcine epithelial cell line LLC-PK1 lacks μ1B and therefore mistargets transferrin receptors to the apical membrane. To determine whether μ1B is involved in basolateral targeting of the $P2Y_1$ receptor, we expressed the receptor in LLC-PK1 cells and determined its localization by confocal microscopy. Just as in MDCK,

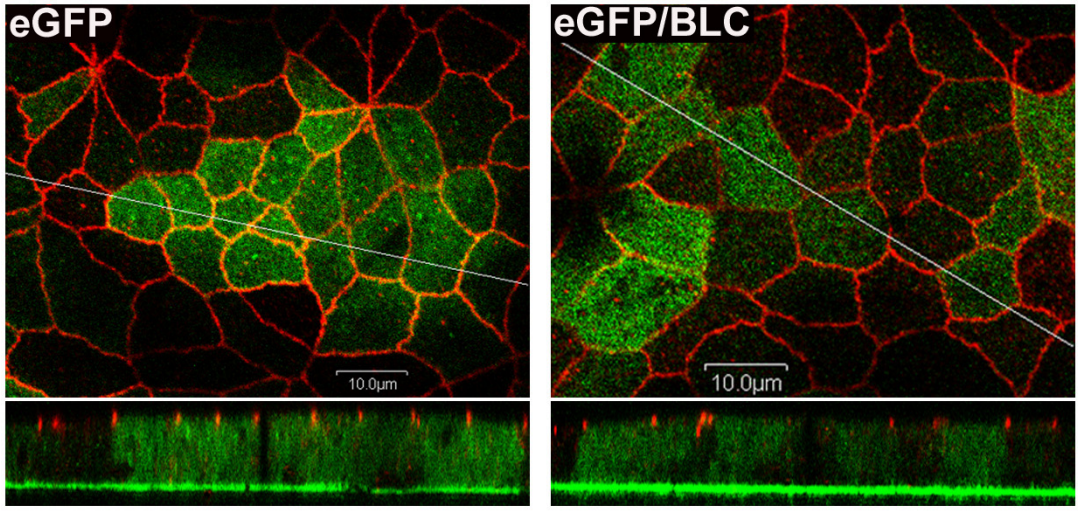


Figure 18. The basolateral cassette (BLC) must be connected to a membrane protein to promote targeting.

The BLC was placed on the C-terminus of green fluorescent protein (GFP) in order to test the ability of the BLC to target a soluble, cytoplasmic protein to the basolateral membrane. GFP with and without the BLC was stably expressed in polarized MDCK(II) epithelia cells and confocal images were collected. Addition of the basolateral cassette to the C-terminus end of GFP did not redirect this soluble protein to the BL membrane surface. This result demonstrated that the BLC must be tethered to the plasma membrane in order to function.

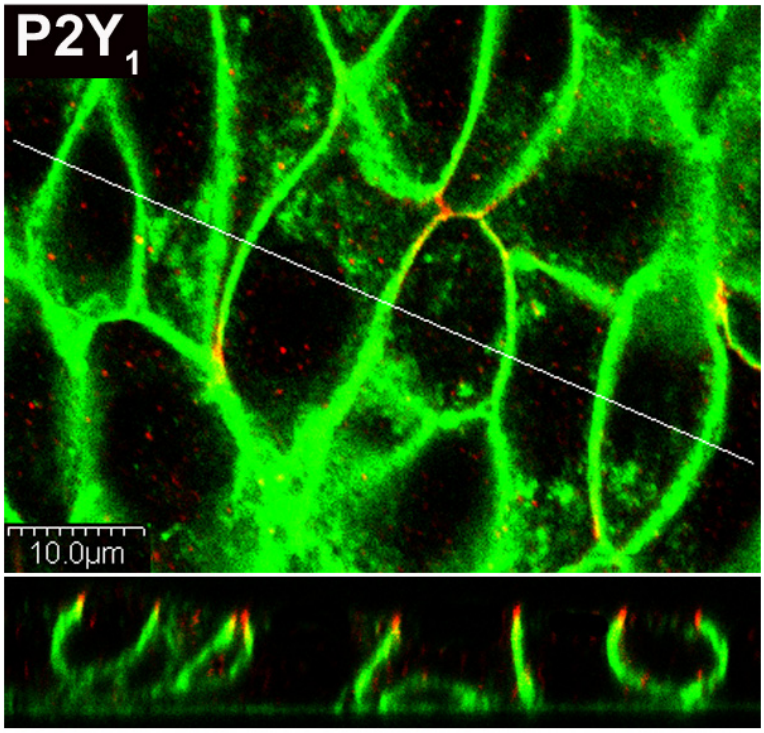


Figure 19. Localization of $P2Y_1$ receptors in LLC-PK1 epithelial cells.

The µ1B subunit of adaptor protein 1 (AP1) has been shown to be involved in the targeting of proteins to the basolateral membrane of polarized epithelial cells. To test the involvement of µ1B in the targeting of $P2Y_1$ to the BL membrane we expressed this receptor in LLC-PK1 epithelial cells, a cell line known to be deficient in µ1B protein, and examined these cells with confocal microscopy.

16HBe14o- and Caco-2 cells, the $P2Y_1$ receptor was expressed exclusively at the basolateral membrane of LLC-PK1 cells (Fig. 19). Thus, μ1B is not involved in basolateral targeting of the $P2Y_1$ receptor.

Several short sequences, such as the tyrosine- and di-hydrophobic-based motifs (Brown and Breton, 2000), have been suggested to act as basolateral targeting motifs. The C-terminal tail of the $P2Y_1$ receptor does not contain a Tyr residue, but there are three potential di-hydrophobic sequences: $L^{350}Q^{351}$, $L^{359}N^{360}$, and $I^{361}L^{362}$. To examine whether di-leucine motifs play any role in targeting, we mutated all of the leucine and isoleucine residues to alanine in the $P2Y_1$-364Z receptor. When expressed in MDCK(II) cells, the mutant receptor was completely basolateral, indicating that di-hydrophobic motifs are not involved in the targeting of $P2Y_1$ receptors (Fig. 20).

3.3 Charged amino acids in the BLC are critical for basolateral targeting

Mutagenesis of the BLC was carried out to identify key amino acids involved in basolateral targeting. Given the length of the signal and the gradual loss of basolateral targeting upon truncation, mutating one or two residues at a time to alanine was likely to be not very informative; therefore, we utilized a more global approach in which we mutated entire functional classes of amino acids to alanine. For example, to determine the role of hydroxylated amino acids (and the potential role of phosphorylation) in targeting, we mutated all five Ser/Thr residues within the BLC and expressed the receptor in MDCK(II) cells. Confocal microscopy revealed no change in basolateral localization, indicating that these amino acids (and by inference, phosphorylation) were not important for targeting of

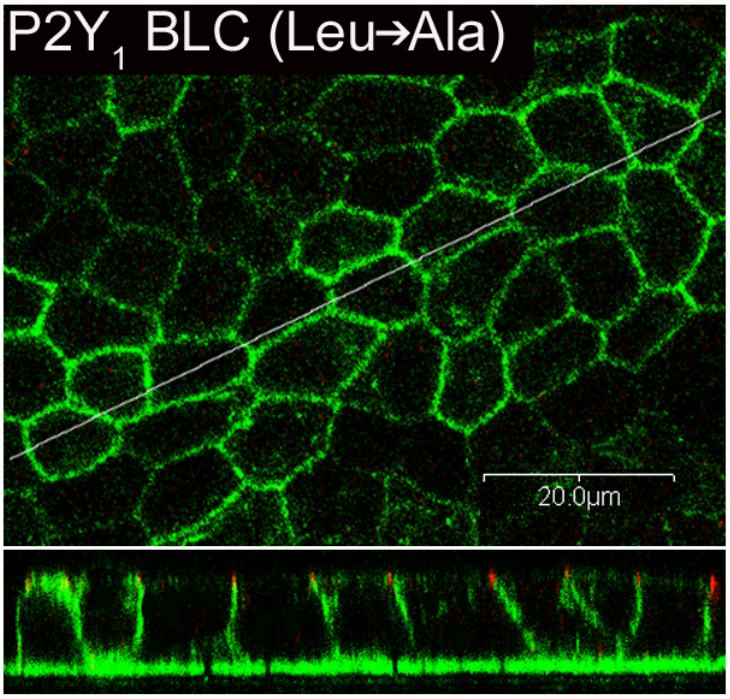


Figure 20. Sorting of the $P2Y_1$ receptor does not utilize a Di-leucine motif.

Di-leucine motifs have been shown to be involved in the targeting of proteins to the basolateral membrane in polarized epithelia. We tested the possibility that the basolateral cassette of $P2Y_1$ may utilize leucine residues in order to function by mutating all four of these amino acids to alanine. This mutant receptor was expressed in polarized MDCK(II) cells and analyzed by confocal microscopy in order to see if mutating leucine residues to alanine disrupted basolateral targeting. The basolateral localization for the $P2Y_1$ BLC (L→A) mutant receptor in MDCK(II) cells indicates that the targeting of $P2Y_1$ to the BL surface does not involve a Di-leucine motif.

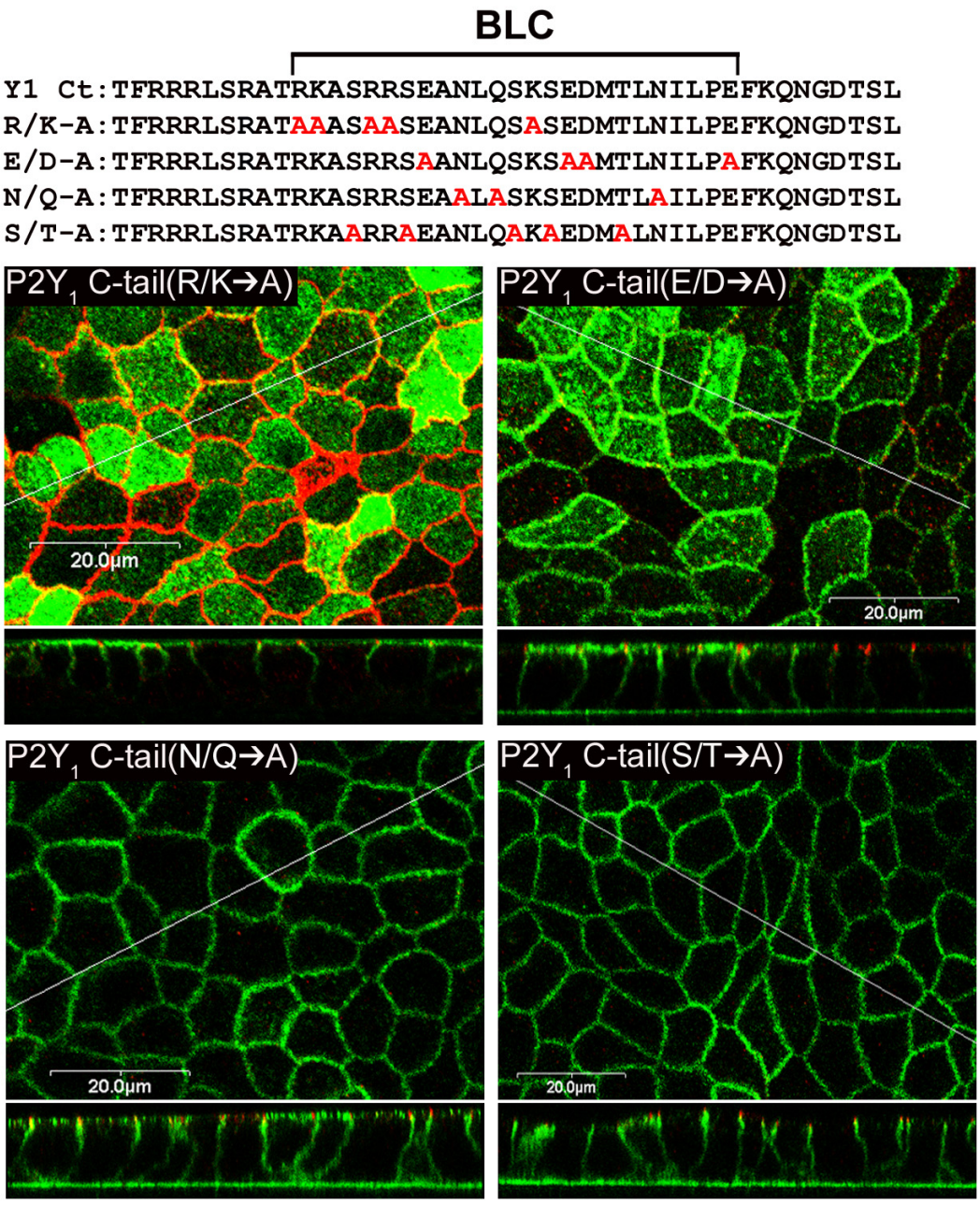


Figure 21. Localization of $P2Y_1$ C-tail mutant receptors.

Receptors containing mutations of the four different classes of amino acids to alanine residues (mutations highlighted in red within primary sequences above) were constructed and stably expressed in polarized MDCK cells. The localization of these mutated receptors was analyzed by confocal microscopy in order to discern which amino acids mediate the basolateral targeting of the $P2Y_1$ receptor.

the $P2Y_1$ receptor (Fig. 21). Mutation of the four amidated amino acids resulted in only a small disruption in basolateral targeting. In contrast, mutation of either all five basic residues or all four acidic residues had much more marked effects on basolateral targeting. For example, mutation of the acidic residues resulted in redirection of nearly 70% of the receptors to the apical membrane, while mutation of the basic residues caused nearly 90% of receptors to be localized to the apical membrane. These data indicated a major role for charged amino acids in basolateral targeting of the $P2Y_1$ receptor. Because the mutations were carried out with full-length protein, one concern was that residues flanking the BLC (especially the charged residues) might influence targeting of the receptor once residues in the BLC were mutated. However, when we repeated these experiments in the context of the $P2Y_2$-315/BLC receptor, which minimizes the concern of flanking amino acids, essentially identical results were obtained (data not shown).

We also examined the targeting properties of the skate $P2Y_1$ ($sP2Y_1$) receptor, which is ~60% identical to its human homologue, to determine whether basolateral targeting was conserved across species, and by extension, whether conserved amino acid residues and/or motifs within its C-terminal tail might mediate such targeting. Comparison of the C-terminal tail sequences for human and skate $P2Y_1$ receptors showed that most of the charged (both basic and acidic) and polar amino acids are conserved (Fig 22). When expressed in MDCK(II) cells, the $sP2Y_1$ receptor was targeted exclusively to the basolateral membrane (Fig. 22). These data are consistent with the conclusion that charged amino acids are critical mediators of basolateral targeting of the $P2Y_1$ receptor.

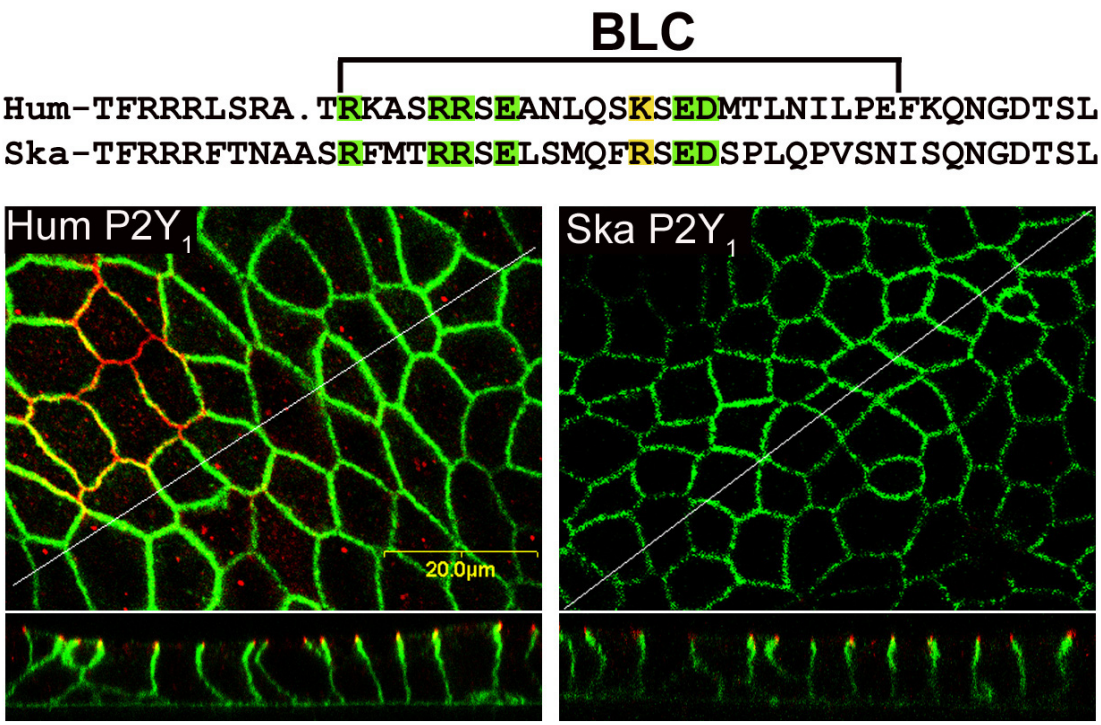


Figure 22. Localization of $P2Y_1$ receptor homologues.

The wild-type human (hum) and skate (ska) $P2Y_1$ receptor homologues were stably expressed in polarized MDCK(II) cells and localization of these tagged receptors was examined by confocal microscopy. These two homologues have very high protein sequence identity except in the C-tail (the location of the BL targeting signal). The skate $P2Y_1$ receptor homologue is clearly localized to the basolateral membrane of MDCK(II) cells as is its human counterpart. This result further emphasizes the critical role of acidic and basic residues in mediating the polarized localization of the $P2Y_1$ receptor (see Figure 21).

3.4 The BLC functions in a sequence-independent manner

The existence of tyrosine- and di-hydrophobic-based basolateral sorting signals in other proteins suggests that at least some basolateral signals have sequence specificity. However, there are many examples of sorted proteins that lack these signals, and within this class of proteins no consensus sequence has been identified to date (Beau et al., 2004; Hobert et al., 1997; Le Gall et al., 1997). To determine the sequence specificity of the BLC, we inverted the BLC (from RKAS…ILPE to EPLI…SAKR) in the context of the full-length $P2Y_1$ receptor and expressed the mutant receptor in MDCK(II) cells. Confocal microscopy and biotinylation revealed that the mutant receptor, like the native $P2Y_1$ receptor, localized almost exclusively at the basolateral membrane (Fig. 23, left panel). These data demonstrate that the BLC is functional, no matter its direction within the C-terminal tail.

One potential explanation for these results is that the BLC forms an amphipathic helix, such that inverting the sequence does not change the character of the BLC. Indeed, examination of the sequence of the BLC by a variety of secondary sequence prediction programs suggested the sequence forms a helix with most of the charged amino acids on one side of the helix and the uncharged amino acids on the other. We tested this hypothesis by scrambling the sequence of the BLC, making sure that the scrambled sequences destroyed any potential amphipathicity of the cassette. As shown in Fig. 23 (middle and right panels), $P2Y_1$ receptors bearing the scrambled BLC sequences were still sorted largely to the basolateral surface. Since disruption of the targeting signal should redirect the protein to the apical membrane by virtue of the apical targeting signal in the main body of the receptor,

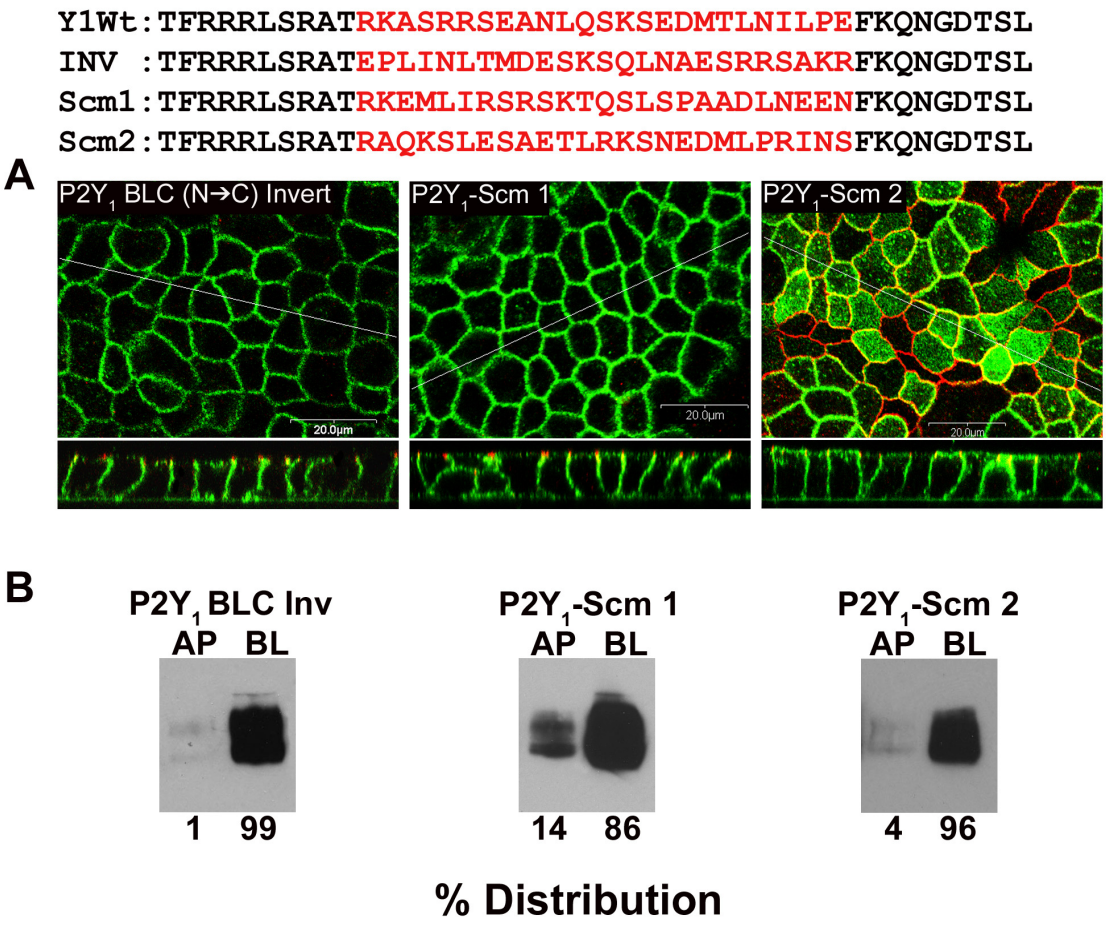


Figure 23. Localization of $P2Y_1$ C-tail inversion and scramble mutant receptors.

A) The role of charged amino acid position in its relation to BLC function was tested by inverting or randomly scrambling (2 versions) the primary sequence of the $P2Y_1$ receptor BLC, expressing these mutants in MDCK(II) cells and analyzing them with confocal microscopy. **B)** Cell surface biotinylation assays were carried out as described in Section 2.4 of Chapter II. The numbers below each lane represent the average percent distribution of the indicated receptor at each membrane domain (n=3).

these data demonstrate that the scrambled sequences were still capable of directing a majority of the protein to the basolateral surface and suggest that the BLC operates largely in a sequence-independent manner.

3.5 The role of charge number and charge balance in basolateral targeting

The data presented thus far suggest that charged amino acids are critical for proper targeting. To investigate the role of charged residues in basolateral targeting in more depth, we made a series of mutations in the BLC of the $P2Y_1$-364Z receptor. This truncated construct was chosen as a template to minimize any problems of charged residues following the BLC substituting for those mutated within the cassette. There are 4 acidic and 5 basic residues within the 25 amino acid BLC, resulting in an overall charge of +1. In the first series of constructs, we progressively mutated pairs of basic and acidic amino acids to alanine, starting from the ends of the cassette and moving towards the middle, while keeping the overall charge of the BLC at +1 (Fig. 24). These receptors were expressed in MDCK(II) cells and their localization was determined by confocal microscopy. Mutation of one or two pairs of charged amino acids (maintaining the overall charge at +1) had a small effect on basolateral targeting (Fig. 24). In contrast, mutation of three pairs of charged amino acids resulted in a severe disruption of targeting, while mutation of four pairs of charged residues resulted in the complete loss of basolateral targeting. These data support a model in which overall charge is critical in maintaining the ability of the BLC to target the $P2Y_1$ receptor to the basolateral membrane. When either the overall charge is altered or the total number of charged amino acids is reduced below a certain threshold, the BLC no longer functions to

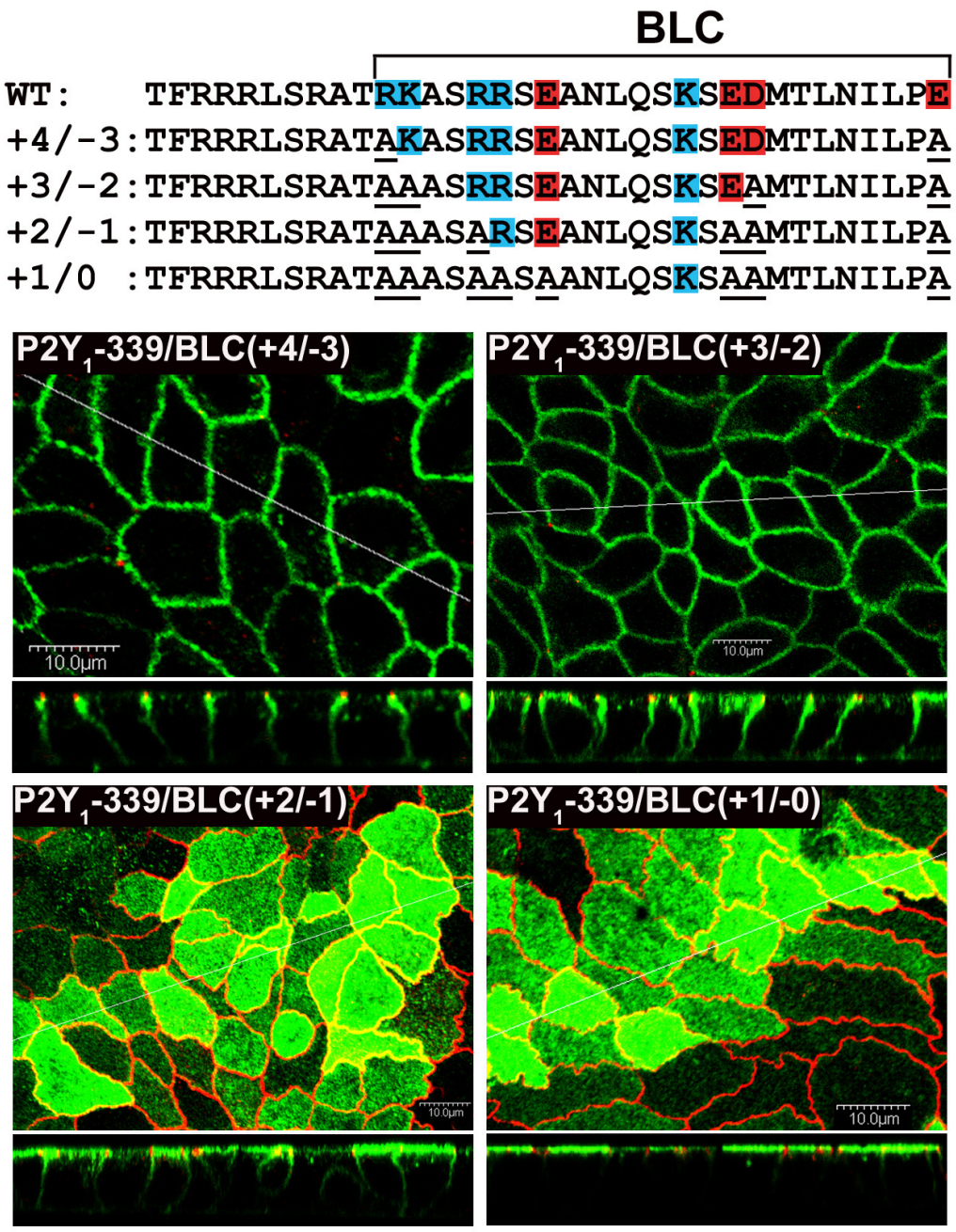


Figure 24. The role of charge number in BLC function.

The number of charged (positive and negative) amino acids in the BLC were reduced by mutating them to alanine (mutant BLC sequences listed above), while maintaining the overall charge of +1. Mutant receptors were expressed in MDCK(II) cells with their targeting profile analyzed by confocal microscopy. These mutant receptors take advantage of the fact that as the targeting function of the BLC is disrupted the receptor moves to the apical membrane, which is easily discerned by confocal analysis.

direct targeting.

In the previous experiment, we examined the role of charge number in BLC function by progressively reducing the number of basic and acidic residues while maintaining an overall balance of +1. The role of charge balance was addressed by maintaining the number of charged amino acids at nine, but shifting the balance to either all negative (i.e. by mutating the basic residues to Asp or Glu) or all positive (i.e. by mutating the acidic residues to Lys or Arg). As shown in Fig. 25, both mutant receptors were localized to the basolateral membrane when expressed in MDCK(II) cells, indicating that a specific balance of charged (positive and negative) amino acids is not required for the proper functioning of the BLC. To be sure that charged residues upstream of the BLC in the $P2Y_1$ receptor were not influencing our results, we also repeated these experiments in the $P2Y_2$-315/BLC construct. The all acidic and all basic BLC constructs behaved identically to their counterparts in the $P2Y_1$ receptor backbone (data not shown), again indicating that the BLC functions as an autonomous signal and does not depend on additional sequences from the rest of the receptor to function.

Finally, we addressed whether the charged and amidated amino acids of the BLC by themselves were necessary and sufficient to direct basolateral targeting. This was tested by mutating all of the uncharged amino acids (Ser, Thr, Leu, Ile, Pro, and Met) to alanine within the BLC of the $P2Y_1$ Δ 364 receptor. As shown in Fig. 26, the mutant receptor was localized to both the apical and basolateral membranes of MDCK(II) cells, demonstrating that the targeting function of the BLC was partially disrupted. These results suggest that the function of the BLC targeting signal is not dependent solely on the amount of charged amino acids

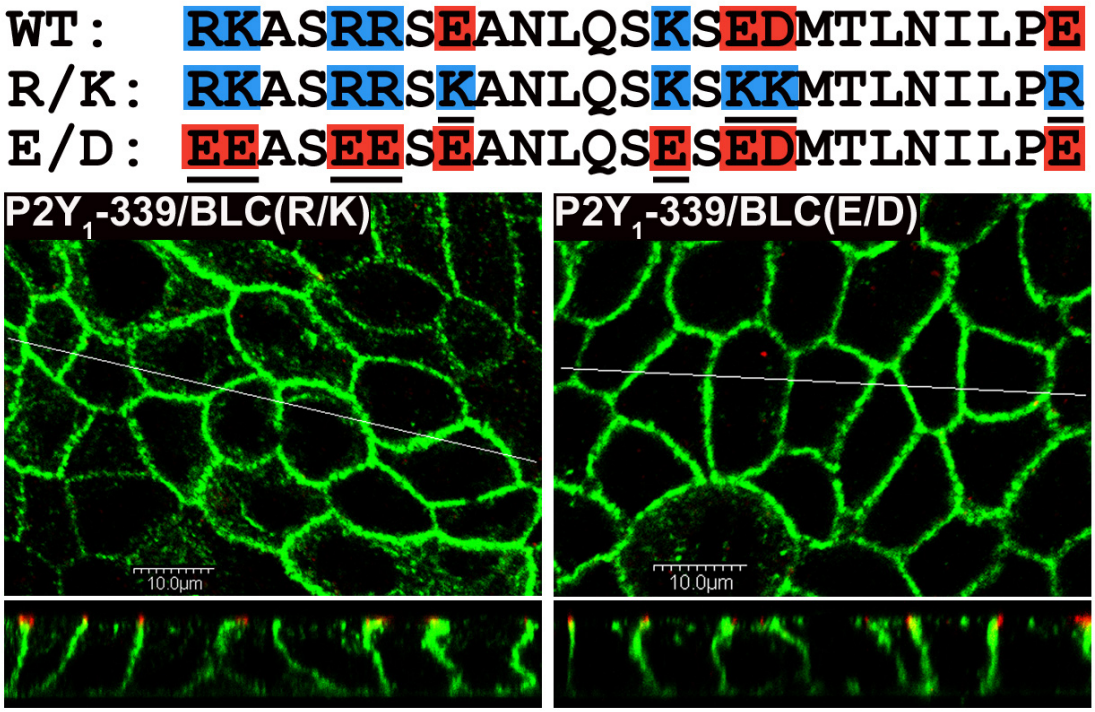


Figure 25. The role of charge balance in BLC function.

The role of overall charge balance in the functioning of the BLC was tested by shifting the wild-type balance from +1 to +9 or -9. This was accomplished by mutating all the basic amino acids within the BLC to acidic residues resulting in the -9 mutant or vice-versa resulting in the +9 mutant (primary sequences listed above). The resulting mutant receptors were expressed in MDCK(II) epithelial cells for confocal analysis and once again took advantage of the fact that disruption in BLC function results in apical targeting, which is easily seen.

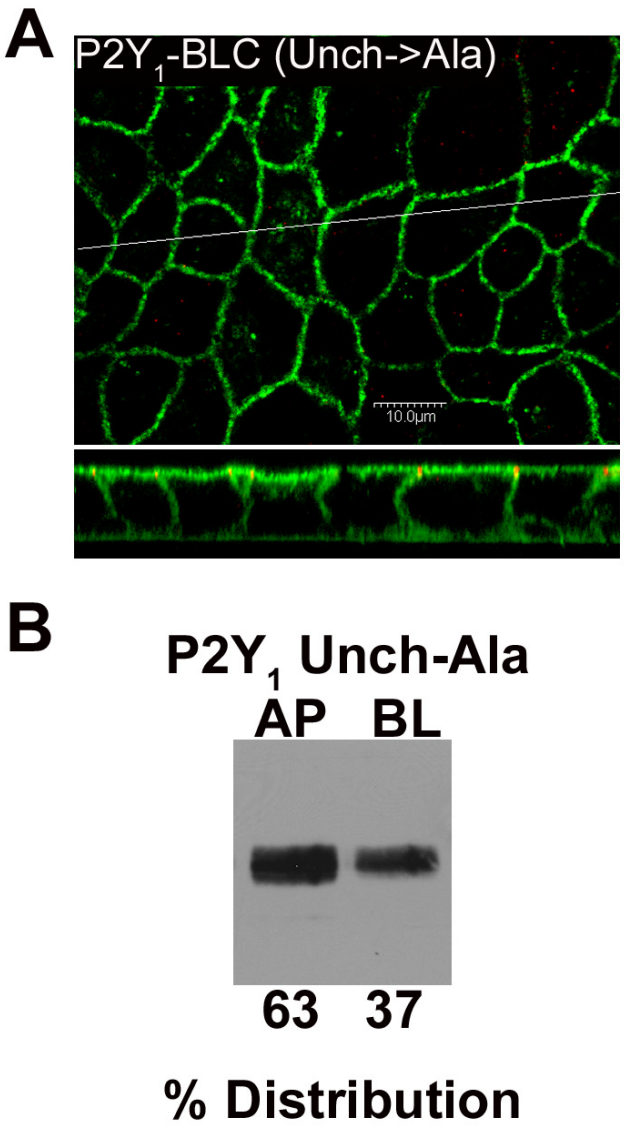


Figure 26. The role of charged/amidated amino acids in BLC function.

A) The role of just the charged and amidated amino acids to direct BL targeting was tested by mutating all other residues (Ser, Thr, Leu, Ile, Met, and Pro) to alanine resulting in the $P2Y_1$ BLC (Unch->Ala) mutant receptor. This mutant receptor was expressed in MDCK(II) **B)** Cell surface biotinylation assays were carried out as described in Section 2.4 of Chapter II. The numbers below each lane represent the average percent distribution of the indicated receptor at each membrane domain (n=3).

present, but has additional requirement(s) as well. One potential interpretation of these results is that mutagenesis of all uncharged amino acids in the BLC to alanine disrupted the secondary structure of the cassette that is critical for a fully functional targeting signal.

4. Discussion

In this study, we have defined several unusual features of the basolateral sorting signal in the C-terminal tail of the $P2Y_1$ receptor. This signal is relatively large (25 amino acids) compared to other basolateral signals, is markedly hydrophilic (9 charged residues), and lacks all previously identified basolateral targeting motifs. Importantly, the signal functions autonomously, as it is capable of redirecting several different receptors to the basolateral membrane provided that it is located close to the end of TM7. Total charge, but not charge balance, within the signal is critical for proper basolateral targeting. Finally, our results indicate that although the signal functions irrespective of the order of the amino acids within the sequence, there likely are structural constraints that are important for proper targeting. These properties have not been recognized for any functional basolateral signal sequence to date.

Basolateral sorting signals characterized to date are usually short, cytoplasmic sequences that can be classified into one of three groups. The first group is characterized by an essential tyrosine that is often part of an NPXY (where X is any amino acid) or YXXΦ motif (where Φ is a bulky hydrophobic residue), the second group by a di-hydrophobic (typically dileucine) sequence, while the third group is comprised of a diverse collection of sequences that vary in length and have no similarities to the targeting signals of the first two groups. The BLC in the $P2Y_1$ receptor, which does not contain a tyrosine-based or di-

hydrophobic motif, is a representative member of the third group of basolateral targeting signals.

Although it is targeted exclusively to the basolateral surface of polarized epithelial cells, the $P2Y_1$ receptor actually contains two targeting signals: an apical signal in the main body of the receptor and the dominant basolateral targeting signal in the C-tail that we have characterized in this study. The presence of two targeting signals in a 7TM receptor has been observed previously. For example, the follicle-stimulating hormone receptor (FSH receptor), which is normally located on the basolateral surface of polarized epithelial cells, is redirected to the apical membrane upon removal of its C-tail (Beau et al., 1998). Thus, there is precedent that a receptor contains two independent targeting signals. In both the $P2Y_1$ and FSH receptor, the basolateral signal is dominant over the apical signal, which is usually but not always observed in other sorted proteins (Mostov et al., 2000). One possibility for the presence of two different signals is that alternative splicing could remove the signal in the C-tail of the receptor, thus allowing the apical signal to act unimpeded. Indeed, the FSH receptor has two alternatively spliced forms in addition to the primary transcript, one of which results in a different C-terminal tail lacking the basolateral targeting signal identified by Beau *et al*. (Beau et al., 1998; Touyz et al., 2000). Although the targeting of this alternatively spliced receptor has not been examined, it remains feasible that splicing may generate forms that target to two different membrane surfaces in Sertoli cells. In contrast, the gene encoding the $P2Y_1$ receptor appears to lack introns, suggesting that this receptor does not undergo alternative splicing. Another possibility is that a primordial receptor was originally targeted to the apical membrane, but during evolution to the current receptor the basolateral signal was created to divert it to the basolateral membrane.

Our investigation into the targeting properties that underpin this unusual basolateral signal yielded some surprising results. First, we investigated the possibility that the BLC operates in a similar fashion to known basolateral sorting signals (i.e. tyrosine or di-leucine based motifs) but found this not to be the case. Once it was established that the basolateral delivery of the $P2Y_1$ receptor was not dependent on previously characterized sorting signals and/or mechanisms we proceeded to determine the properties of this novel signal. Several different experiments were carried out showing that the signal is a 25 amino acid cassette in which the cluster of charged residues are most critical for proper function. Furthermore, the signal requires that it be tethered to a membrane-bound protein and be in close proximity to TM7 in order to function effectively. Moreover, the BLC is not dependent on a specific sequence nor does it require a certain balance of positively and negatively charged amino acids but seems to rely solely on charge number.

Surprisingly, we showed that sequence specificity of the BLC is not required for its function. Thus, the function of the BLC is almost entirely retained when the sequence was inverted C→N, scrambled (two different scrambled sequences were tested), or when the charged residues were changed to either all positive or all negative. These results strongly suggest that the signal functions on the basis of a physiochemical interaction (i.e. general hydrophilicity) and not by a specific protein:protein binding scheme or salt-bridge interactions. One of our initial hypotheses on the mechanism of the basolateral sorting function of the BLC was that the positive charges (perhaps localized to one side of a helix) interact with the negatively charged phospholipid surface to promote the association of the receptor with a particular lipid microenvironment found only in the basolateral membrane. This hypothesis was similar to the mechanism described for the MacMARCKS protein, a

myristoylated PKC substrate and peripheral membrane protein (Myat et al., 1998). Aderem and colleagues have shown that a cluster of positively charged amino acids in MacMARCKS promotes association to the basolateral membrane of MDCK(II) epithelial cells, presumably through an electrostatic interaction with negatively-charged phospholipids located in the inner leaflet of the lateral plasma membrane. Lateral localization is then lost when the protein is phosphorylated by PKC, which neutralizes the positively charged signal and disrupts the electrostatic interaction between MacMARCKS and the plasma membrane. However, our data clearly show that the BLC of the $P2Y_1$ receptor functions normally even when all of the basic residues are mutated to acidic residues, which would likely rule out any interaction with negatively charged phospholipids. Thus, the $P2Y_1$ receptor BLC peptide must direct basolateral targeting by a different mechanism.

We hypothesized that the $P2Y_1$ basolateral targeting signal and the way it functions is not an isolated example in nature but characterizes a new type or class of sorting signal that heretofore has not been described. We explored this hypothesis by searching for any published reports describing an autonomous basolateral signal that did not operate according to any known motifs/mechanisms and could not be fully explained by the primary investigators. Several examples were found scattered throughout the literature and include two members of the epidermal growth factor receptor family (EGFR/ErbB1 and ErbB2), the neural cell adhesion molecule (N-CAM), and thyrotropin stimulating hormone receptor (TSHR) and are listed (including the $P2Y_1$ receptor) in table 4. With the exception of the ErbB2 receptor, these targeting signals are approximately 20 amino acids in length and contain a cluster of both negatively and positively charged residues that comprise at least

Receptor	Primary Sequence	Chrg/Tot	Reference
ErbB2	LQ**E**T**E**LV**E**PLT	3/11 (27%)	(Dillon et al., 2002)
EGF	**KR**TL**RR**LLQ**ERE**LV**E**PLTPSG**E**A	9/23 (39%)	(Hobert et al., 1997)
N-CAM	PN**HD**GG**KH**T**E**PN**E**TTPLT**E**	7/19 (37%)	(Le Gall et al., 1997)
TSHR	**R**QGL**H**NM**ED**VY**E**LI**E**NS**H**	7/18 (39%)	(Beau et al., 2004)
$P2Y_1$	**RK**AS**RR**S**E**ANLQS**K**S**ED**MTLNILP**E**	9/25 (36%)	

Table 4. Basolateral targeting signals with unidentified sorting motifs.
A search of the literature reveals several basolateral targeting signals that were successfully identified and delimited, but do not contain any previously characterized motifs. The primary sequence for each of these signals is listed with the charged amino acids in bold. In addition, the number of charged residues over the total number of amino acids is provided, which is utilized to calculate the charge percentage for each signal.

35% of the signal. In addition, all of these targeting signals are located in close proximity to the plasma membrane.

Interestingly, the EGF (ErbB1) and ErbB2 receptors belong to the same family but utilize different portions of the same basolateral targeting signal in order to achieve a polarized distribution. In the case of the EGFR, a long version that is 23 amino acids confers basolateral targeting, while a shorter portion (11 amino acids) of the same exact signal is all that is required for the basolateral targeting of the ErbB2 receptor (see alignment in Table 4). The authors of the ErbB2 study suggest that these overlapping findings provide strong evidence that this juxtamembrane region of the receptor contains critical targeting information.

Published reports on the ErbB2, N-CAM, and EGFR basolateral targeting signals identified key amino acids that mediated signal function to varying degrees. In the case of the EGF receptor, the investigators demonstrated that a polyproline core as well as an arginine residue play a role in basolateral targeting, while proline and glumatic acid residues seem to play a role in directing basolateral targeting in the N-CAM sorting signal. However, in both cases the mutagenesis of these key amino acids only caused partial disruption to the basolateral targeting signal. In all four examples (ErbB2, N-CAM, TSHR and EGFR) the investigators were able to delimit the targeting signals but were unable to identify all of the key amino acids involved in the functioning of these signals suggesting that other residue(s) are involved. One interpretation of these results is that a critical mass of charged amino acids is required for proper receptor targeting to the basolateral membrane.

Although total charge is the predominant feature directing basolateral targeting of the $P2Y_1$ receptor, our data and analyses also indicate that is not the only requirement. For

example, the C-tail of the apically targeted $P2Y_6$ receptor is 23 amino acids long and contains 11 charged residues in close proximity to the plasma membrane (**KK**F**RRR**P**HE**LLQ**K**LTA**K**WQ**R**QG**R**). If total charge was all that was important, the $P2Y_6$ receptor might be expected to be basolaterally localized, but this is clearly not the case. This observation strongly suggests that other features in addition to a cluster of charged amino acids exist for these peptide sequences to act as targeting signals. One possibility is that there exists a conformational requirement (i.e. secondary structure) for these peptides to confer targeting information. Consistent with this possibility, when we mutated all of the uncharged and non-amidated amino acids within the BLC to alanine while keeping the charged and amidated amino acids intact, basolateral targeting was markedly disrupted. This suggests that in addition to total charge, some structural information is also important for proper targeting. The predominantly basolateral location of the $P2Y_1$ receptor containing a scrambled BLC may reflect the fact that this structural information is mostly retained when the amino acids were scrambled. Indeed, several studies have suggested that a beta-turn or other secondary structural motif is required for proper targeting by cytoplasmic signals (Aroeti et al., 1993; Beau et al., 2004; Choowongkomon et al., 2005).

So how does this sequence direct basolateral targeting? Our data discount a protein that binds in a sequence-specific manner, but perhaps there are sorting proteins that recognize structural features such as a critical mass of charged residues. These proteins might bind to these sorting signals in a charge-dependent, sequence-independent manner and direct sorting to the proper membrane surface. In this manner, these proteins would act in a similar fashion as β-arrestins, which bind to the C-terminal tails of multiple GPCRs in a phosphorylation-dependent, sequence-independent manner, and promote endocytosis through clathrin-coated

pits (Lefkowitz and Whalen, 2004). One such protein could be VPS26, a protein component of the retromer involved in vacuole sorting in yeast, that has a structure highly reminiscent of β-arrestin, including a polar core that is a critical structural feature of arrestins (Shi et al., 2006). Moreover, VPS26 was reported to be involved in transcytosis of the polymeric immunoglobin receptor (pIgR) in polarized epithelial cells (Verges et al., 2004). One intriguing feature of this scenario is that proteins would not have to rely on a specific sequence motif, freeing the sorting signal to interact with other proteins important for the function of the specific receptor after the signal directed transport of the protein to the basolateral surface. Consistent with this idea, calmodulin has been reported to interact with the C-terminus of the $P2Y_1$ receptor, in a region that overlaps with the BLC sequence reported here (Arthur et al., 2006).

In conclusion, we have described a unique basolateral targeting signal that may help to define a new class of sorting signal. Our extensive mutagenesis studies determined that the $P2Y_1$ receptor is targeted to the basolateral membrane domain by a 25 amino acid cassette that relies on charged residues in order to function, must be in close proximity to the PM and operates in an autonomous fashion. The results of this study in combination with the findings of other investigations on autonomous basolateral targeting signals (i.e. EGFR, TSHR and N-CAM) suggest that these signals may be operating under the same principles. Furthermore, our results suggest that this targeting signal must adopt an as-yet unknown secondary structure (e.g. α-helix) in order to function. Indeed, more studies are required in order to substantiate our hypothesis that the $P2Y_1$ receptor basolateral targeting signal defines a new class of sorting signal.

CHAPTER V: The C-terminal tail of the $P2Y_4$ receptor contains an apical targeting signal

1. Introduction

The human $P2Y_4$ receptor is a uracil nucleotide-activated member of the P2Y receptor family that was cloned in 1995 by multiple labs (Communi et al., 1995; Nguyen et al., 1995; Stam et al., 1996) and followed by the cloning of the rat (Bogdanov et al., 1998; Webb et al., 1998) and mouse (Lazarowski et al., 2001; Suarez-Huerta et al., 2001) orthologues. The human orthologue of this receptor is selectively activated by UTP and completely antagonized by ATP, whereas the rodent orthologues are activated equipotently by both ATP and UTP (Herold et al., 2004; Kennedy et al., 2000). The original cloning reports found message for this receptor in human placenta and pancreas tissues, while subsequent publications have reported expression of either mRNA or protein for the $P2Y_4$ receptor in the human lung (Communi et al., 1999), murine stomach, intestine and liver (Suarez-Huerta et al., 2001), and in the epithelium of the gerbil inner ear (Marcus and Scofield, 2001; Sage and Marcus, 2002).

It took several years after the cloning of the $P2Y_4$ receptor before a physiological role could be established. One of the first studies to examine the physiological role of the $P2Y_4$ receptor was by Ko and colleagues, who examined the nucleotide regulation of ion transport in equine epithelial cells (Ko et al., 1997). They observed an increase in I_{sc} when UTP or ATP was applied to the apical surface of these cells, suggesting that P2Y receptors regulated ion transport. Further studies utilizing a cross-desensitization approach revealed the presence

of two distinct P2Y receptor populations, one activated by ATP and UTP (and subsequently identified as the $P2Y_2$ receptor) and a UTP-selective receptor that later was shown to be the $P2Y_4$ receptor. Subsequent studies by this laboratory (McAlroy et al., 2000; Wilson et al., 1998; Wong and Ko, 2002) demonstrated that activation of apically bound $P2Y_2$, $P2Y_4$ or $P2Y_6$ receptors along with the downstream release of intracellular calcium regulates ion transport in equine epithelial cells through a calcium-activated chloride channel (CaCC).

The regulation of epithelial ion transport by $P2Y_4$ receptors was further examined in a number of published reports. One group showed a loss of nucleotide regulation of chloride transport in the jejunum of $P2Y_4$ receptor knock-out mice (Robaye et al., 2003), while others demonstrated that the $P2Y_4$ receptor is involved in the regulation of epithelial potassium current in the mouse colon (Matos et al., 2005) and the gerbil vestibulum (Marcus and Scofield, 2001). Taken together, these studies define the physiological role of the $P2Y_4$ receptor, at least in epithelial cells, as a regulator of ion transport, one of the most important functions of this cell type (Leipziger, 2003).

The studies described above not only helped to establish a physiological role for the $P2Y_4$ receptor but they also strongly suggested a polarized distribution of this receptor to the apical membrane domain of epithelial cells. While the pharmacological and physiological evidence was convincing, no one had published any studies showing an apical targeting pattern with a direct method such as immunofluorescence, nor had any targeting signal been identified. Thus, we utilized confocal microscopy to directly visualize and confirm the apical localization of $P2Y_4$ receptors in a variety of epithelial cells (described in Chapter II) (Wolff et al., 2005). Once the steady-state localization pattern of all the P2Y receptors was established, we determined the location of the targeting signals for all the receptors

displaying a polarized (i.e. apical or basolateral) distribution pattern (described in Chapter III), including the $P2Y_4$ receptor. The focus of this chapter is characterization of a novel apical targeting signal located in the C-tail of the $P2Y_4$ receptor.

2. Materials and methods

2.1 Construction of HA-tagged $P2Y_4$ and BK_2 receptor chimeras, mutants and truncations

Three general methods were used to generate the HA-tagged P2Y receptor chimeras, truncations, and mutant constructs utilized in this study. These methods are described in Chapter IV section 2.1 and were utilized to construct HA-tagged $P2Y_4$ and BK_2 receptor chimeras, mutants and truncations, which are described in this chapter.

2.2 Cell culture and expression of receptor constructs

Madin-Darby canine kidney type II cells (MDCK(II); ATCC, Rockville, Maryland) were subcultured in DMEM/F12 (1:1) medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS; Hyclone, Gaithersburg, MD) and 1X pen/strep in a humidified incubator at 37^{o}C with 5% CO_2 and 95% air.

Recombinant retroviral particles were produced by calcium phosphate-mediated transfection of PA317 cells with pLXSN vectors containing HA-tagged hP2Y receptor constructs as previously described (Comstock et al., 1997) and used to infect the cell lines listed above. Geneticin-resistant cells were selected after 7-10 days with 1 mg/ml G418 and maintained in medium containing 0.4 mg/ml G418.

2.3 Confocal Microscopy

This assay was performed as described in Chapter II section 2.3 under the same heading.

2.4 Quantification of cell surface HA-tagged P2Y receptor constructs

This assay was performed as described in Chapter II section 2.4 under the same heading.

3. Results

3.1 Delimiting the apical targeting signal in the $P2Y_4$ receptor C-tail

As shown in section 3.2 of Chapter III, the $P2Y_4$ receptor contains a single targeting signal within its C-tail that directs the receptor to the apical membrane of MDCK(II) cells (Fig. 12 in Chapter III). In order to delimit this signal, we constructed two series of truncations/deletions in its C-tail to delimit the N-terminal and C-terminal ends of the signal. These experiments took advantage of the fact that once the apical targeting signal is removed, the receptor becomes unsorted. We defined the C-terminal end of the apical sorting signal by truncating the last 9 ($P2Y_4$-355Z), 21 ($P2Y_4$-343Z) or 32 ($P2Y_4$-332Z) amino acids from the C-terminal end of the full length $P2Y_4$ receptor, and expressing these truncated receptors in MDCK(II) for confocal analysis. As seen in Figure 27, the first two truncated receptors ($P2Y_4$ Δ355 and $P2Y_4$ Δ343) were targeted exclusively to the apical membrane, while the third truncated receptor ($P2Y_4$ Δ332) was unsorted. These results demarcate the C-

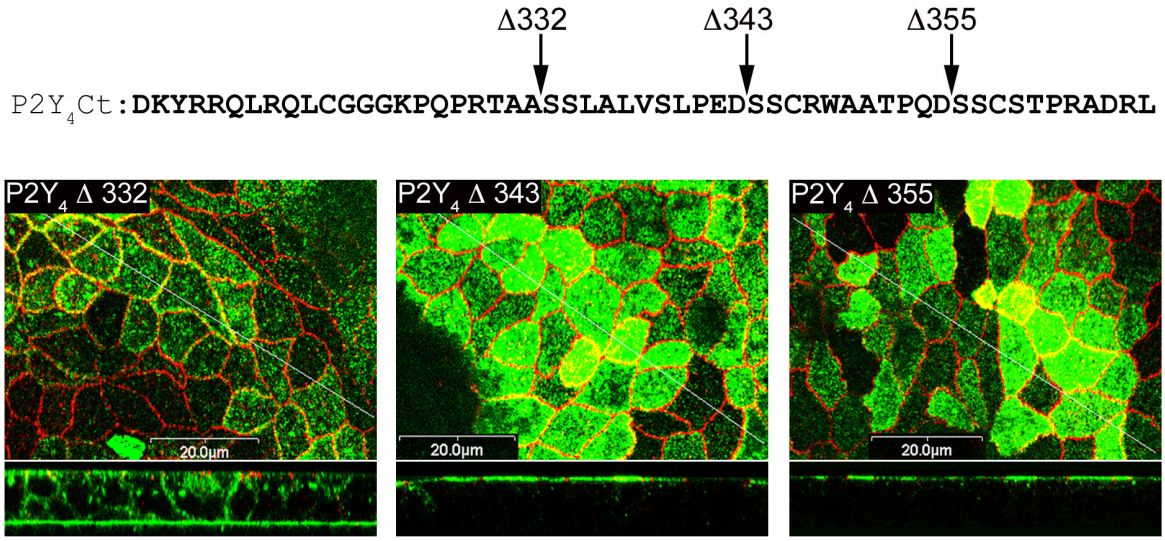


Figure 27. Defining the C-terminal end of the $P2Y_4$ receptor apical signal.

The C-terminal end of the $P2Y_4$ receptor apical targeting signal was defined through a series of truncation experiments. These experiments took advantage of the fact that once the apical targeting signal is removed, the receptor becomes unsorted. We removed the last 9 ($P2Y_4$ Δ 332), 21 ($P2Y_4$ Δ 343), or 32 ($P2Y_4$ Δ 355) amino acids starting from the C-terminal end of the full length $P2Y_4$ receptor. Confocal analysis of these truncated receptors demonstrate that the C-terminal end of this apical signal is located at Asp^{343}.

terminal end of the $P2Y_4$ apical signal as Asp^{343}. To determine the N-terminal end of the apical sorting signal, we replaced the endogenous C-tail of the unsorted BK_2 receptor with progressively shorter versions of the $P2Y_4$ C-tail with all them ending at Asp^{343}, resulting in the construction of $BK_2/P2Y_4$ C-tail @(^{311}DKYR…PED^{343}), $BK_2/P2Y_4$ C-tail@(^{316}QLRQ…PED^{343}), $BK_2/P2Y_4$ C-tail@(^{321}CGGG…PED^{343}), or $BK_2/P2Y_4$ C-tail@(^{326}KPQP…PED^{343}) chimeric receptors. Confocal analysis of this series showed that the first three receptors (^{311}DKYR…PED^{343}, ^{316}QLRQ…PED^{343}, and ^{321}CGGG…PED^{343}) were localized to the apical membrane, whereas the ^{326}KPQP…PED^{343} receptor was unsorted, demonstrating that the N-terminal end of the $P2Y_4$ apical signal is located at Cys^{321} (Fig. 28). Taken together, these series of experiments define the apical targeting signal as a 23-amino acid cassette with the following primary sequence: ^{321}CGGGKPQPRTAASSLALVSLPED^{343}.

3.2 The apical targeting cassette (APC) confers targeting on a basolateral receptor

One of the hallmarks of a strong sorting signal is its ability to impart a specific targeting itinerary to either an unsorted protein or a protein with a different localization pattern. Therefore, we tested whether the APC of the $P2Y_4$ receptor C-tail was capable of imparting apical localization to the $P2Y_{12}$ receptor, which is expressed at the basolateral surface even when its C-tail is removed. For these studies, the $P2Y_4$ receptor C-tail from ^{311}DKYR…PED^{343} was fused to the truncated $P2Y_{12}$ receptor just past TM7 to create the $P2Y_{12}/P2Y_4$ C-tail chimera. Confocal analysis showed an almost completed redirection of the $P2Y_{12}$ receptor to the apical domain, demonstrating that the APC is both necessary and

BK_2/Y_4@DKYR:**KRFRDKYRRQLRQLCGGGKPQPRTAASSLALVSLPED**
BK_2/Y_4@QLRQ:**KRFRQLRQLCGGGKPQPRTAASSLALVSLPED**
BK_2/Y_4@CGGG:**KRFRCGGGKPQPRTAASSLALVSLPED**
BK_2/Y_4@KPQP:**KRFRKPQPRTAASSLALVSLPED**

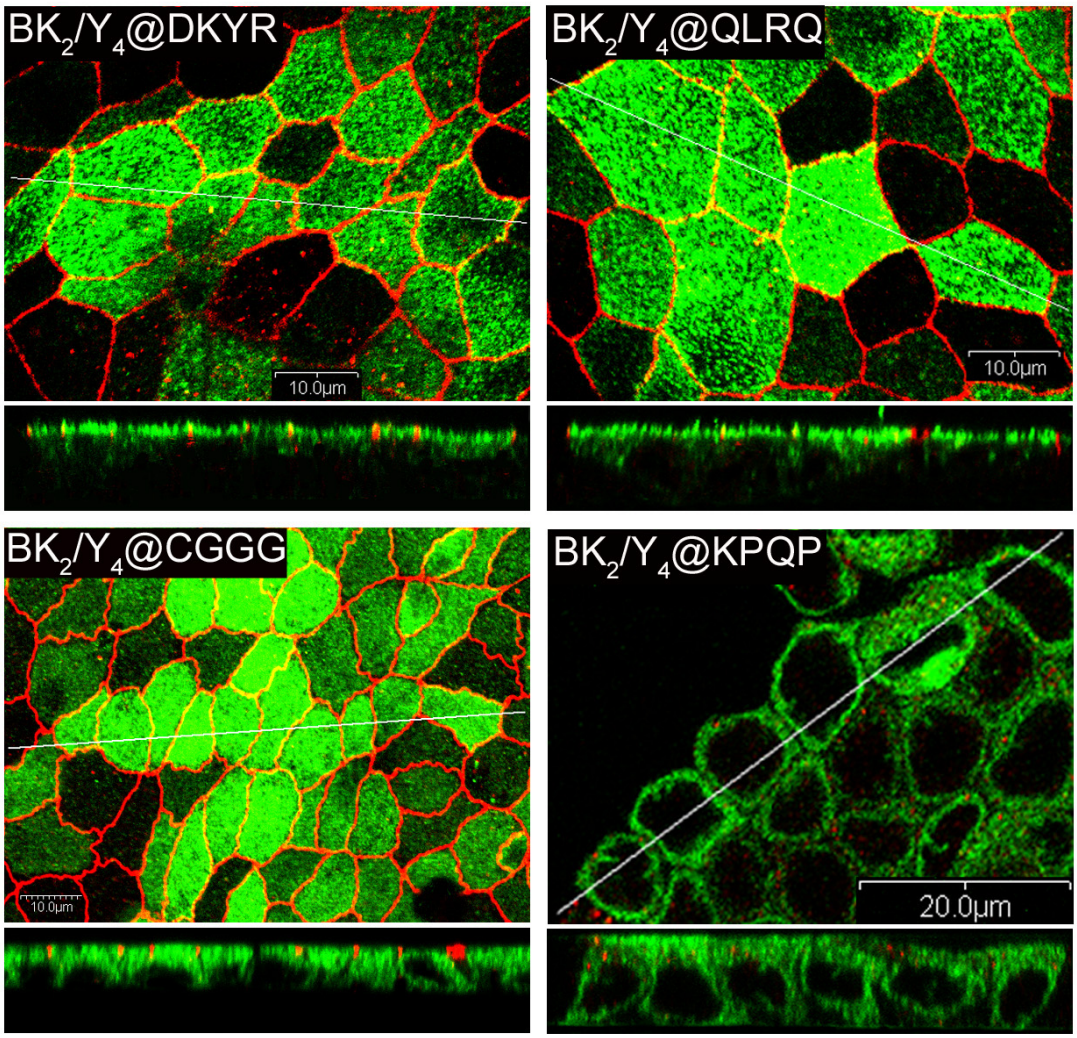


Figure 28. Defining the N-terminal end of the apical targeting signal.

The N-terminal end of the apical targeting cassette (APC) was defined by replacing the endogenous C-tail of the unsorted BK_2 receptor with progressively shorter versions of the $P2Y_4$ receptor C-tail with all of them ending at Asp^{343}. The primary sequence of each mutant is listed above. Confocal analysis of this series of mutants defined the APC as a 23 amino-acid cassette with the following sequence: **CGGGKPQPRTAASSLALVSLPED.**

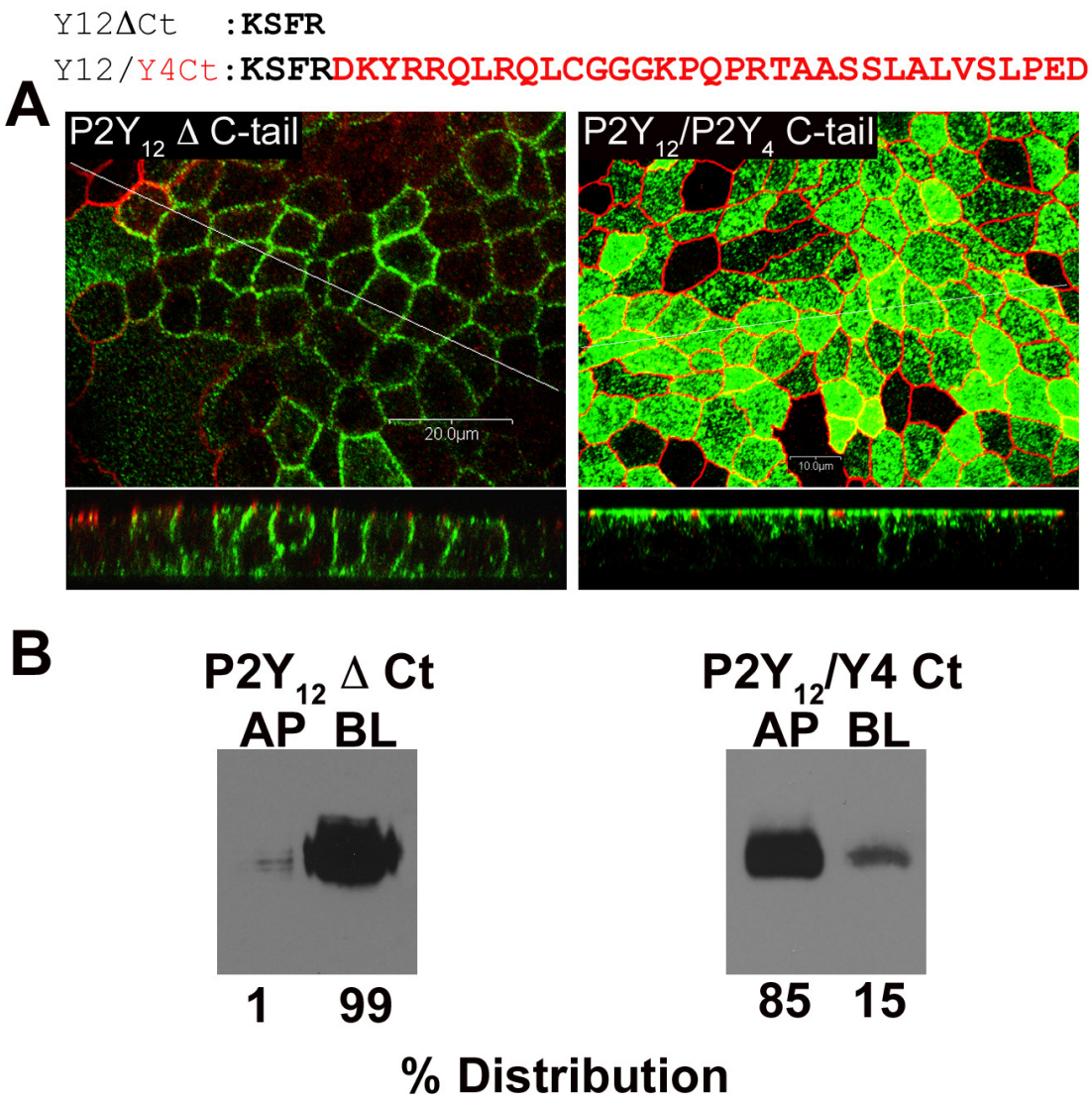


Figure 29. The apical targeting cassette (APC) confers targeting on a basolaterally-sorted $P2Y_{12}$ receptor.

A) The truncated form of the $P2Y_{12}$ receptor missing its C-tail ($P2Y_{12}$ Δ C-tail) is targeted to the basolateral membrane of MDCK(II) cells (as shown above) as is its wild-type counterpart, making it an ideal backbone to test the strength of apical targeting signals. The strength of the APC was tested by constructing a $P2Y_{12}/P2Y_4$ C-tail chimera (primary sequence of C-tail region shown above), expressing it in MDCK(II) epithelial cells and examining the localization of the receptor with confocal microscopy. **B)** Cell surface biotinylation assays were carried out as described in Section 2.4 of Chapter II. The numbers below each lane represent the average percent distribution of the indicated receptor at each membrane domain (n=3).

sufficient to confer apical targeting (Fig. 29) and that it is dominant over a basolateral signal. These results are in contrast to the dogma in the targeting field that basolateral signals always override apical signals.

3.3 Inversion of the APC does not disrupt apical targeting

In Chapter IV, we demonstrated that the $P2Y_1$ receptor basolateral targeting signal functions whether it is in its normal N→C orientation or when inverted in the C→N orientation. This result led us to address whether orientation of the $P2Y_4$ receptor APC is crucial to its function. The 23 amino acid APC was inverted from a N→C to C→N orientation within the context of the $BK_2/P2Y_4$ C-tail chimera, and the resulting receptor was examined by confocal microscopy for alterations in its targeting properties. As seen in Figure 30, the $BK_2/P2Y_4$ C-tail (Inv) receptor remains mostly at the apical surface with some cytoplasmic staining seen below the plasma membrane, perhaps suggesting that the receptor is not as stable as other chimeras. Overall, we concluded that inversion of the APC had little to no effect on its ability to target receptors to the apical membrane but probably decreases the stability of the receptor at the plasma membrane.

3.4 The potential role of palmitoylation and phosphorylation in APC function

Palmitoylation or acylation of proteins can be crucial for their trafficking to and stability at the plasma membrane (Linder and Deschenes, 2007), while phosphorylation also has been shown to regulate the cell surface expression of proteins (Mandela and Ordway, 2006; Myat et al., 1998). We constructed two mutant receptors to test the potential role for

BK2/Y4Ct :**KRFRDKYRRQLRQLCGGGKPQPRTAASSLALVSLPED**
BK2/Y4Inv:**KRFRDKYRRQLRQLDEPLSVLALSSAATRPQPKGGGC**

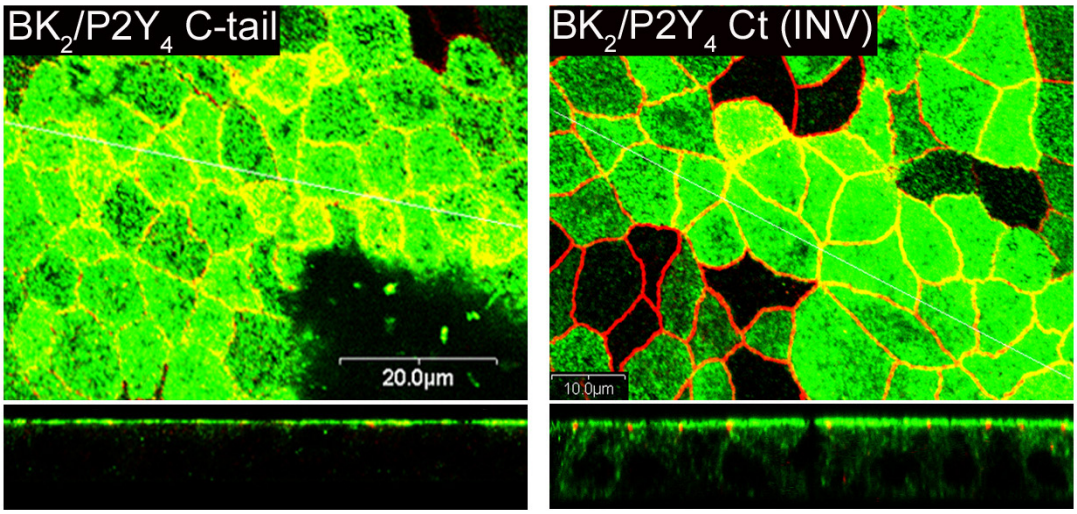


Figure 30. Inversion of the APC has a minor effect on its functioning.

Previous experiments on the $P2Y_1$ receptor targeting signal addressed whether inverting this signal would have any effect on its function. We carried out the same experiment on the APC of the $P2Y_4$ receptor to test whether orientation of the signal's primary sequence played a role in its function. The $BK_2/P2Y_4$ C-tail chimera is localized to the apical membrane of MDCK(II) cells demonstrating the proper functioning of the APC, thus, making it an ideal chimera to carry out these experiments. The 23 amino acid APC was inverted from its normal N→C to a C→N orientation (inverted sequence is underlined) within the context of the $BK_2/P2Y_4$ C-tail receptor, expressed in MDCK(II) cells and examined with confocal microscopy for any changes in targeting.

these protein modifications in the functioning of the APC. In the first mutant receptor, a potential palmitylation site in the APC (Cys^{321}) was eliminated by mutating this amino acid to Ser (resulting in the hBK2/$P2Y_4$ C-tail C321A mutant), while the second mutant examined the role of hydroxylated amino acids (and the potential influence of phosphorylation) by mutating all of the Ser and Thr residues within the entire C-tail of the $P2Y_4$ receptor to alanine (resulting in the $P2Y_4$ C-tail S/T-A mutant). As shown in Figure 31, both mutant receptors were expressed at the apical domain, suggesting that the APC functions independently of both these potential modifications.

3.5 Mutagenesis analysis of the apical targeting signal

The next series of experiments were designed to identify key amino acids involved in the functioning of this apical targeting cassette (APC) by utilizing a mutagenesis approach. With this approach we constructed four different mutant receptors and expressed them in MDCK(II) cells for examination of their targeting properties by confocal microscopy. Experiments characterizing the BLC in the $P2Y_1$ receptor demonstrated that charged amino acids are critical to signal function; therefore, we tested the role of acidic and basic amino acids in the functioning of the APC by mutating relevant residues to alanine. In addition, we wanted to test the role of a hydrophobic core located near the C-terminal end of the signal by mutating the hydrophobic residues (Leu and Val) to alanine. The final construct tested the role of potential secondary protein structure created by the $^{326}PQP^{328}$ motif by mutating these amino acids to alanine (resulting in the hBK2/$P2Y_4$ Ct PQP→AAA mutant). Confocal

C321S:**QLRQLSGGGKPQPRTAASSLALVSLPEDSSCRWAATPQDSSCSTPRADRL**
S/T-A:**QLRQLCGGGKPQPRTAAAALALVALPEDAACRWAAAPQDAACAAPRADRL**

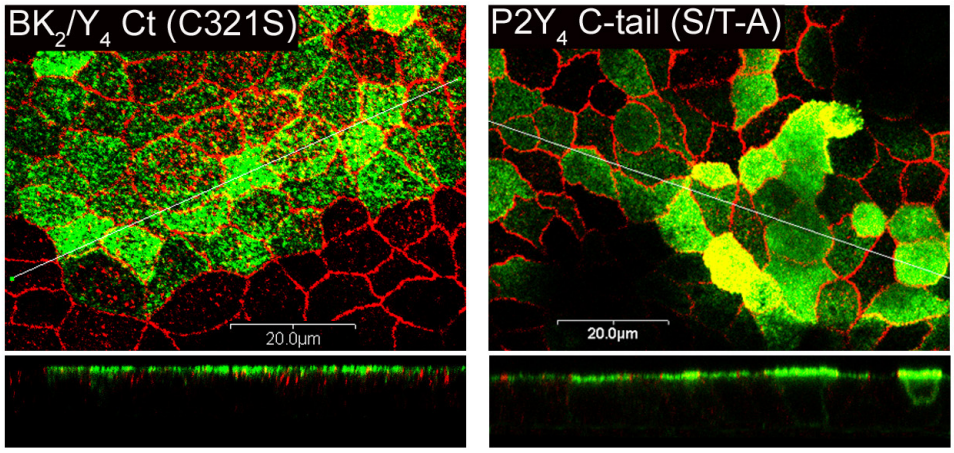


Figure 31. Potential role for acylation and phosphorylation in APC function.

Both palmitoylation and phosphorylation of proteins regulate their delivery to the plasma membrane and may play a vital role in the targeting of the $P2Y_4$ receptor. We tested this hypothesis by mutating the target amino acids for both palmitate and kinases to inert residues and looked for disruptions in apical targeting, which would indicate a role for these modifications in APC function. In the case of palmitoylation, Cys^{321} was mutated to serine (mutation underlined), while the role of phophorylation was investigated by mutating all of the serines and threonines (with exception of a single Thr) to alanine (mutations underlined).

analysis of the targeting profile for these mutant constructs yielded some surprising results. In all four mutants, there was no significant disruption in apical targeting of the receptor, demonstrating that these different classes of amino acids and potential secondary structure are not involved in the functioning of the APC (Fig. 32). However, we did observe significant accumulation of receptor in the cytoplasm of the cell especially in the case of the mutant hydrophobic receptor (L/V→A). This result in particular seems to suggest that the APC may do more than act as cellular zip code ensuring proper delivery to the apical membrane of epithelial cells. One possibility includes the APC acting as a membrane or scaffolding anchor, ensuring stability of the receptor once it is delivered to the apical domain.

4. Discussion

In this chapter we have described a series of experiments to delimit and characterize the apical targeting signal in the C-tail of the $P2Y_4$ receptor. We determined that the apical signal is a 23 amino acid linear cassette (primary sequence: ^{321}CGGGKPQPRTAASSLALVSLPED^{343}) that is capable of overriding a basolateral sorting signal in another P2Y receptor and does not require modifications such as palmitoylation or phosphorylation in order to function. In addition, this apical targeting cassette (APC) remains capable of operating as a sorting signal upon inversion of its primary sequence or after mutation of its charged residues (i.e. K/R→A and E/D→A). Taken in its entirety, the mutagenesis results seem to suggest that the APC does not rely on a few key residues for its function as is the case for other signals (Cheng et al., 2002), but requires the entire sequence for proper operation. Furthermore, the results also seem to suggest the APC may have a role

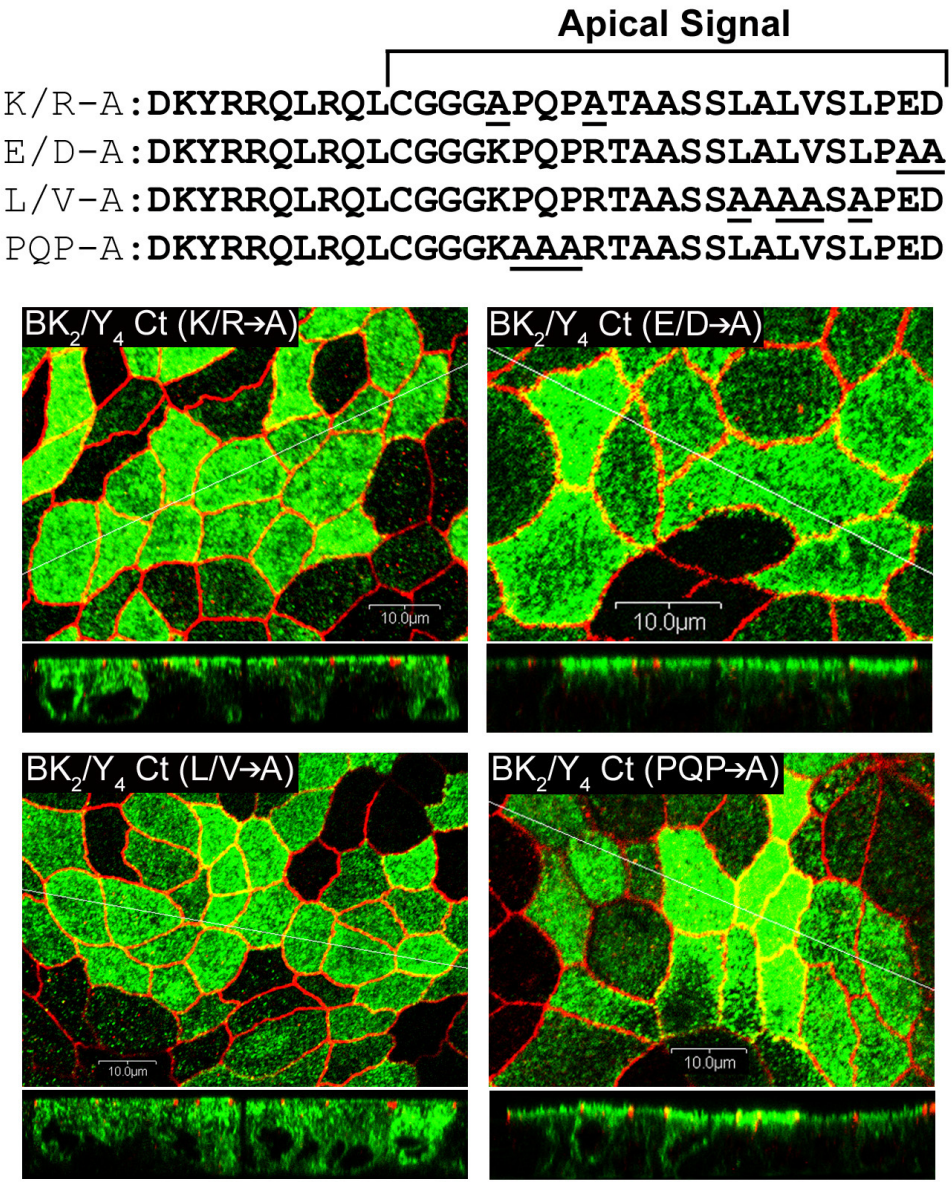


Figure 32. Mutagenesis analysis of the APC.

A series of mutagenesis experiments were carried out in order to identify key amino acids, if any, that are involved in the functioning of the APC. These mutageneis experiments were carried out in the context of the apically targeted $BK_2/P2Y_4$ C-tail chimera wherein we tested the role of basic (K/R→A) and acidic (E/D→A) amino acids as well as hydrophobic residues (L/V→A) by mutating them to alanine as indicated. Finally, we mutated a short proline motif (PQP) to alanine to test the potential role a secondary structure may play in APC function given the fact that prolines may induce such structure.

in protein stability due to the major disruption in expression of cell-surface receptor by the L/V→A mutant. The elucidation of this new sorting signal adds to the growing list of similar signals (discussed below) and may help to uncover a consensus sequence or physical properties important for targeting of proteins to the apical membrane.

Basolateral targeting signals are usually short peptide sequences located in the cytoplasmic region of proteins that have been shown, in some cases, to interact with specific sorting machinery (i.e. AP1) to ensure proper delivery with high-fidelity (Mostov et al., 1999). Furthermore, consensus basolateral targeting sequences have emerged from the last 15 years of research that include tyrosine- and dileucine-based motifs (Mostov et al., 2003). In the case of apical targeting signals and related sorting machinery, the picture is less clear. These signals have been found in the extracellular, transmembrane and intracellular (cytoplasmic) regions of proteins, and until very recently little progress has been made on identifying the machinery involved in apical targeting. The initial work on identifying apical targeting signals determined that post-translational modifications such as glycosylation was sufficient to confer apical targeting to at least some proteins, presumably by allowing the modified protein to associate with lipid rafts at the apical domain (Rodriguez-Boulan and Gonzalez, 1999). However, glycosylation does not automatically shuttle a protein to the apical membrane and is therefore not considered to be a *bona fide* sorting signal.

Our understanding of apical targeting (i.e. the signals and machinery involved) was recently expanded when several labs published reports describing novel apical targeting signals together with possible mechanisms by which the signals may operate. The first of these identified a linear apical targeting signal within the C-tail of the rhodopsin GPCR (Chuang and Sung, 1998). More specifically, these investigators delineated a 32 amino acid

signal (MLTTICCGKNPLGDDEASATVSKTETSQVAPA) that was necessary and sufficient to confer apical targeting to a protein normally targeted to the basolateral membrane. Furthermore, they showed that palmitoylation of the signal was not required for function. A follow up study by this group in 2001 showed that the rhodopsin apical sorting signal interacts with cytoplasmic dynein, a minus-end microtubule motor protein, strongly suggesting that movement along the cell's cytoskeleton via a specific motor protein is the mechanism by which rhodopsin achieves apical localization (Tai et al., 2001). Other published reports also have contributed valuable information. For example, a report by Amara and co-workers elucidated a targeting signal that directs a specific excitatory amino acid transporter (EAAT3) to both the apical membrane of MDCK(II) epithelial cells and equivalent domain (dendrities) of hippocampal neurons (Cheng et al., 2002). In an elegant series of experiments they delimited the signal to an 11 amino acid cassette (KSY**VN**GG**F**AVD) and identified three key residues (in bold) that mediate its function as a targeting signal. Most recently, an 11 amino acid sequence (PTPPTVENQQR) was identified as a necessary and sufficient signal to sort guanylyl cyclase C (GCC) to the apical membrane of MDCK(II) epithelial cells (Hodson et al., 2006). Unfortunately, key residues involved in the functioning of this signal could not be identified.

In the present study, an apical targeting signal located in C-tail of the $P2Y_4$ receptor seemingly shares little similarity with other sorting signals in the same class. What similarity that does exist between these signals lies in the topology of their primary sequences in that they all contain relatively few charged amino acids and, in 3 out of the 4 sequences, contain proline residues that could impart important secondary structural constraints to the signal. Finally, both the rhodopsin and $P2Y_4$ receptor targeting signals do not require palmitoylation

in order to operate. Other investigators have conducted similar analyses of apical targeting signals in hopes of elucidating a common motif but have yielded no consensus (Cheng et al., 2002).

In spite of the progress that has been made on the discovery of new apical targeting signals, consensus sequences such as those identified for basolateral targeting have yet to emerge. Some have speculated that the diversity of these signals reflects the diversity of pathways that target proteins to the apical domain (Cheng et al., 2002; Hodson et al., 2006). As more of these targeting signals are identified along with possible binding partners, a more complete and clear understanding of the apical targeting machinery will certainly follow.

CHAPTER VI: General Conclusions and Future Directions

The physiological role of P2Y receptors is diverse considering their nearly ubiquitous expression throughout the body. One role that has been unequivocally demonstrated for these receptors is the regulation of ion transport in epithelial cells. Kohn and co-workers published one of the first experiments suggesting purinergic-regulation of ion transport in 1970, where they demonstrated ATP stimulation of ion transport in rat small intestine (Kohn et al., 1970). Since these first reports, the role of P2Y receptors in epithelial ion transport has been extensively studied, which have shown that epithelial cells from a variety tissues express at least one or more P2Y receptors (reviewed in (Leipziger, 2003)). Furthermore, it has been hypothesized that purinergic receptors mediate numerous physiological processes in epithelia due to the fact that release of nucleotides (i.e. ATP and UTP) is metabolically 'inexpensive' and thus provides a very effective system that responds to cellular events such as stress (Insel et al., 2001).

In addition to establishing a physiological role for purinergic receptors in epithelia, many studies presented evidence suggesting a polarized distribution of these receptors as well. For example, a series of studies by Ko and collaborators demonstrated the apical localization of the uridine-activated $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in polarized equine sweat epithelia (Wilson et al., 1998; Wong and Ko, 2002), while Insel's group has shown the polarized distribution of the $P2Y_1$, $P2Y_2$ and $P2Y_{11}$ receptors in MDCK(II) epithelial cells (Insel et al., 2001; Zambon et al., 2001; Zambon et al., 2000). With the exception of a single

published report by Zambon *et al* on the targeting of the canine $P2Y_{11}$ receptor (Zambon et al., 2001), all the studies examining the localization of P2Y receptors in epithelial cells utilized indirect methodology such as the measurement of apically- or basolaterally-induced I_{sc}.

It was this lack of direct evidence demonstrating the steady-state distribution of the P2Y receptor family in polarized epithelial cells that served as a starting point for this dissertation research. Utilizing direct experimental techniques (confocal microscopy and biotinylation assay) we successfully established the distribution pattern of all eight members of this receptor family, which is described in Chapter II of this dissertation. Interestingly, we determined that seven of the eight members of the P2YR family are localized to either the apical or basolateral membrane surface. Once the steady-state distribution of the entire P2YR family was established, we next investigated the location of the targeting signal(s) for each receptor subtype that drives the protein to either the apical or basolateral membrane. We found that all seven polarized receptors contain at least one targeting signal that is necessary and sufficient to confer polarized targeting (Chapter III). Finally, the basolateral and apical targeting signals located in the respective C-tails of the $P2Y_1$ and $P2Y_4$ receptor were fully characterized as described in Chapters IV and V, respectively. We conclude that P2Y receptors must play a critical role in the functioning of epithelial cells given 1) the pervasive expression of these receptors through out numerous epithelial-rich tissues 2) that all but one are distributed in a polarized manner and 3) they utilize a wide range of targeting signals to achieve this distribution.

While the characterization of P2Y receptor targeting signals has been fruitful and engaging, we recognize that in order to continue our success we need to elucidate the

mechanism(s) by which these receptors achieve a polarized distribution in epithelial cells. In order to achieve this goal, several different experimental approaches could be taken. One such experiment is pulse-chase studies, which determine the route of delivery a protein utilizes to arrive at the apical or basolateral membrane domain. Determining this property can potentially provide some important information. For example, the appearance of a receptor at the same membrane domain over a long period of time would indicate direct delivery to this surface. Follow up studies could include treatment with brefeldin A (blocks vesicle budding in the ER) and/or low temperature (prevents vesicles from reaching the PM), which if proven effective in terminating protein delivery would point to the use of a classical secretory pathway. This pathway utilizes transport vesicles that originate in the TGN and are passed along a variety of compartments before reaching a specific membrane domain where protein-containing vesicles successfully deliver their cargo. This information would prove quite valuable because many of these pathways are fairly well characterized in that we know many of the proteins that bind to the vesicles and their cargo in order to achieve polarized targeting. For example, the exocyst is a protein complex that ferries numerous proteins to the basolateral membrane and have a finite amount of binding partners. Ultimately, the goal of these experiments would be to find potential binding partners for P2Y receptors and begin to unravel the mystery as to how this large family travels to either the apical or basolateral membrane. In addition, other methods could be used to find potential binding partners including yeast-2-hybridization screens.

In the case of the $P2Y_1$ BLC, we have characterized a most unusual and unique targeting signal in that it operates by having a critical mass of charged amino acids that does not require a specific sequence. The non-specificity of the signal is quite intriguing and

suggest a couple of potential explanations that might be worthy of investigation. Clearly, our data discount a protein that binds in a sequence-specific manner, but perhaps there are sorting proteins that recognize structural features such as a critical mass of charged residues. These proteins might bind to these sorting signals in a charge-dependent, sequence-independent manner and direct sorting to the proper membrane surface. In this manner, these proteins would act in a similar fashion as β-arrestins, which bind to the C-terminal tails of multiple GPCRs in a phosphorylation-dependent, sequence-independent manner, and promote endocytosis through clathrin-coated pits (Lefkowitz and Whalen, 2004). One such protein could be VPS26, a protein component of the retromer involved in vacuole sorting in yeast, that has a structure highly reminiscent of β-arrestin, including a polar core that is a critical structural feature of arrestins (Shi et al., 2006). Moreover, VPS26 was reported to be involved in transcytosis of the polymeric immunoglobin receptor (pIgR) in polarized epithelial cells (Verges et al., 2004). One intriguing feature of this scenario is that proteins would not have to rely on a specific sequence motif, freeing the sorting signal to interact with other proteins important for the function of the specific receptor after the signal directed transport of the protein to the basolateral surface.

Another intriguing hypothesis is that the $P2Y_1$ C-tail mediates BL targeting by interacting with lipids, which is quite feasible given the prominence of lipid rafts in MDCK(II) cells in organizing signaling proteins within the plasma membrane (Scheiffele et al., 1998). Furthermore, unpublished results from this laboratory and others demonstrate that the $P2Y_1$ receptor floats in the lighter fractions of a sucrose gradient in a caveolin-1 dependent manner, suggesting that the receptor associates with lipid rafts. Therefore, future directions include investigating the potential role of caveolae/lipid rafts in P2YR targeting

via several approaches including the use of a caveolin-1 dominant-negative that has been shown to ablate the formation of caveolae (Lee et al., 2002; Razani et al., 2002). We are curious as to the effect this dominant negative would have on the basolateral or apical targeting of the respective $P2Y_1$ and $P2Y_2$ receptors.

The most exciting possibility is that through careful analysis of the $P2Y_1$ receptor BLC we have uncovered a new class of targeting signal. In the discussion section of Chapter IV, we hypothesize that this novel BL targeting signal operates by having a critical mass of charged amino acids in close proximity to the plasma membrane. Moreover, we provide examples from the literature of targeting signals that seem to fit this mold, which serve to bolster our hypothesis. However, only further investigation and a careful eye on the latest targeting publications will allow us to determine the validity of our theory.

Finally, the secondary structure of proteins has been shown to mediate targeting in epithelial cells (Aroeti et al., 1993). Might the $P2Y_1$ BLC form such a structure as part of its function? Prelimary circular dichroism and NMR experiments suggest that the BLC does indeed form an amphipathic helix in presence of membrane-mimicking conditions. Whether this is a naturally occurring phenomena and the impact it may or may not have on the functioning of the signal remain to be seen. Experiments are currently underway to begin to address these questions.

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