University of Alberta

Regulation And Function Of The Protein Inhibitor Of Nitric Oxide Synthase (PIN)/Dynein Light Chain 8 (LC8) In Human Mast Cells

by

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ABSTRACT

The protein inhibitor of nitric oxide synthase (PIN)/dynein light chain 8 (LC8) is responsible for binding numerous proteins and facilitating their transport within cells. One of these proteins has been identified as the neuronal isoform of nitric oxide synthase (nNOS). Nitric oxide (NO) has been shown to regulate many functions in mast cells (MC). We predicted that MC express PIN/LC8, and that its regulation could be influenced by cytokines. We further hypothesized that PIN/LC8 is capable of binding nNOS in MC, as well as other proteins, and that these interactions impact MC function. We found PIN/LC8 mRNA and protein was expressed in MC, and was differentially regulated by T_h1 and T_h2 cytokines. Furthermore, we found PIN/LC8 bound nNOS, as well as other proteins, and interfering with this binding resulted in a NO-independent increase in leukotriene (LT) production. These results indicate that PIN/LC8 is important in MC function.

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5-LO	5 lipoxygenase
A23187	calcium ionophore
aa	amino acid
AAT	α-1-antitrypsin
ANOVA	analysis of variance
ATP	adenosine triphosphate
bp	base pair
BH_4	tetrahydrobiopterin
Ca^{2+}	calcium
CD 34+	cluster of differentiation 34 positive
cDNA	complementary deoxyribonucleic acid
c-kit	receptor for stem cell factor
CLB	cell lysis buffer
cNOS	constitutive NOS
COX	cyclooxygenase
Ct	critical fluorescence threshold
D	aspartate
DAG	diacylglycerol
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
DTT	dithiothreitol
dUTP	deoxyuridine triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
eNOS	endothelial NOS
ER	endoplasmic reticulum
FAM	5'6-carboxyfluorescein
FBS	fetal bovine serum
$Fc_{\varepsilon}RI$	high affinity Fc-epsilon receptor-I
G	glycine
GAPDH	glyceraldehyde phosphate dehydrogenase
GFP	green fluorescent protein
GKAP	guanylate kinase domain-associated protein
GST	glutathione-S-transferase
GTP-CHI	GTP cyclohydrolase I

h	hour
HC	dynein heavy chain
HEK	human embryonic kidney cell line
HMC-1	human mast cell line-1
HSMC	human skin mast cell
HSP-90	heat shock protein 90
I	isoleucine
IC	dynein intermediate chain
IFN-γ	interferon-gamma
IgE	immunoglobulin E
IgG	immunoglobulin G
IκB	inhibitory factor kappa-B
IL-3	interleukin-3
IL-4	interleukin-4
IL-10	interleukin-10
iNOS	inducible nitric oxide synthase
IPTG	isopropyl-β-$_D$-thiogalactopyranoside
IP3	inositol triphosphate
ITAM	immunoreceptor tyrosine activation motif
K	lysine
kDa	kilodalton
L	leucine
LAD-2	laboratory of allergic diseases-2 cell line
LB	luria-bertani medium
LC	dynein light chain
LIC	dynein light-intermediate chain
LC8	dynein light chain 8 kDa
L-NAME	N^G-nitro- L-arginine methyl ester
LPR	late phase response
LT	leukotriene
min	minute
MC	mast cell
MC_C	mast cell chymase positive
MC_T	mast cell tryptase positive
MC_{TC}	mast cell tryptase and chymase positive
MRP-1	multidrug resistance-associated protein 1
MS	mass spectrometry
MT	microtubule
NADPH	nicotinamide-adenine dinucleotide phosphate
NCBI	National Center of Biotechnology Information

NO	nitric oxide
NOS	nitric oxide synthase
NOSIP	eNOS interacting protein
NOSTRIN	eNOS traffic inducer protein
nNOS	neuronal nitric oxide synthase
O^{2-}	superoxide
$ONOO^-$	peroxynitrite
P	proline
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDZ	protein-protein binding domain
PGD	prostaglandin
PIN	protein inhibitor of NOS
PKC	protein kinase C
PLCγ	phospholipase gamma
PLA2	phospholipase A2
PMSF	phenylmethylsulphonyl fluoride
Q	glutamine
Q-tof	quadropole time of flight
R	arginine
RIPA	radioimmunoprecipitation
RT	reverse transcriptase
RyR	ryanodine receptor
s	second
SMC	human skin MC
SR	sacroplasmic reticulum
T	threonine
TBS	tris buffered saline
T_h1	T-helper type 1
T_h2	T-helper type 2
TNF	tumor necrosis factor
UV	ultra violet
V	valine
wk	week
XOR	xanthine oxidoreductase

Chapter 1

General Introduction

Introduction

I. Mast Cells

A. Origin and Development.

Mast cells (MC) arise from pluripotent CD34+ progenitor cells in human bone marrow, where they reach a committed but immature phenotype before exiting into the circulation [1]. These circulating progenitors have a distinct surface molecule expression pattern that is CD 34+, c-kit+, LY-, CD17-, and CD14- [2]. They can enter tissues, migrate to their final tissue destination and complete their maturation under the influence of their new microenvironment [3].

Though stem cell factor is the key driving element in the growth and differentiation of these cells [3,4], many growth factors and cytokines influence growth and differentiation of MC, including IL-3, IL-4, IL-9, IL-10, and nerve growth factor (NGF) [3]. Mature MC contain all the basic organelles, including endoplasmic reticulum (ER), mitochondria, and a large nucleus, but in addition, MC contain a large number of round, membrane bound granules which store various mediators and proteins essential to MC function [5].

B. Heterogeneity

Human MC are heterogeneous in different sites in the body, a feature strongly influenced by effects of microenvironmental factors on their development. Differences can include size, granule structure, and types and amounts of stored mediators [6]. A classical factor which can help identify MC subpopulations is the protease content located in the granules [3]. One group of human MC contain

tryptase, chymase, cathepsin G, and carboxypeptidase and are designated MC_{TC} due to their content of both tryptase and chymase. Another group is known as MC_T, as it only contains tryptase [7], and a minor subgroup is known to only contain chymase (MC_C) [8]. The two main MC subsets differ in their anatomical locations as well. MC_T are largely in the alveolar tissue of lung (~93%) and the mucosa of the small intestine (~80%), whereas MC_{TC} are mainly in the skin (~99%) and intestinal submucosal (~80%) associated [3]. Interestingly, these two MC populations can respond differently to the same stimuli. MC_{TC} from skin are sensitive to stimulation by the neuropeptide substance P, whereas MC_T from the lung are not [3], highlighting the importance of understanding the heterogeneity of these cells.

C. Activation.

There is a wide spectrum of molecules that can activate MC, and these secretagogues employ a number of receptors and signaling pathways to exert their effects. Classically, MC are activated via antigen-mediated cross-linking of IgE molecules, bound to their high affinity receptor ($Fc_{\varepsilon}RI$) on the cell surface [3,9]. Additionally, MC can be activated by various peptides [3], cytokines [3] and charged molecules [10], as well as elements of bacteria, viruses, and parasites [11].

1. IgE-dependent activation.

$Fc_{\varepsilon}RI$ exists as a heterotetramer on the cell surface. It is made up of the IgE binding α-chain, one β-chain, and two disulphide linked γ-chains [12]. The β and γ-chains contain conserved sequences known as immunoreceptor tyrosine-based activation motifs (ITAM) through which they interact with the proximal molecules

involved in the signaling cascade [12]. Aggregation of these receptors results in the release of three categories of mediators from MC: stored granule-associated mediators (including histamine and proteases), lipid-based mediators (such as prostaglandins and leukotrienes), and induced cytokines and chemokines whose expression are induced [13]. Release of the first two mediator groups is rapid, shown to maximize within 5 minutes, whereas production of cytokines and chemokines takes several hours to reach a maximum, given the length of the transcription and translation processes [13].

Once $Fc_{\varepsilon}RI$ is appropriately stimulated, initial signaling events occur that include recruitment and activation of the src family kinase, Lyn. Lyn is able to bind and phosphorylate the β and γ chains of $Fc_{\varepsilon}RI$ on their ITAM regions [12]. This allows for another src family kinase, Syk, to bind to $Fc_{\varepsilon}RI$-γ chains via its src-homology-2 (SH2) domain [14]. When this occurs, Lyn is then able to phosphorylate and activate Syk, a critical step in MC activation [12]. Another src family kinase, Fyn, has been shown to be critical to FcεRI activation as MC from Fyn-null mice are unable to degranulate [15]. Fyn is involved in the activation of the pluripotent protein kinase C (PKC) and phosphatidylinositol 3-OH kinase (PI3K), whose actions are central to MC degranulation.

There are two major branches of this signaling which lead to the release and production of the three classes of mediators mentioned above. The first involves phosphorylation, and thus activation, of phospholipase Cγ (PLCγ) by Syk [12]. PLCγ catalyzes the hydrolysis of phosphatidyl-inositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes release of

intracellular Ca^{2+} stores and DAG co-activates, along with Fyn, PKC [16], and both of these steps are directly involved in immediate granule exocytosis.

Syk is also able to phosphorylate the membrane-anchored linker for activation of T cells [12], which acts like a scaffold to form a mulitmeric signaling complex involving Grb-2, SOS, SLP-76, and VAV [13]. VAV is a guanine exchange factor able to activate Ras, which in turn activates the mitogen-activated protein kinase (MAPK) pathway [13,17]. MAPK pathway activation, along with increased intracellular calcium, regulates phospholipase A_2 (PLA_2) which initiates the enzymatic cascade leading to lipid-derived mediator production and release [18]. Additionally, downstream targets of both PKC and the MAPK proteins are transcription factors, and are responsible for regulating the synthesis of cytokines and chemokines in MC [12,13].

2. IgE-independent activation.

MC can also be activated by a number of substances in a $Fc_{\varepsilon}RI$ independent fashion. This includes substance P, the effector domain of Rab 3A, neutrophil defensin [3,19,20], and polybasic molecules, such as mastoparan, 48/80, and polymyxin B [10]. It appears that there are many shared characteristics between these two groups, including their method of causing histamine release from MC. Currently, it appears there are no specific receptors for either of these groups of secretagogues [19,22] apart from substance P. A recent study has shown that a NK1 receptor antagonist prevented stress-induced degranulation in rat PMC [23],

suggesting that under certain conditions the NK1 receptor is required for substance P to stimulate MC.

The essential feature common to both groups for the action of these molecules is a cationic cluster next to a hydrophobic region. For example, mastoparan and mellitin both display three basic amino acids and a separate large hydrophobic domain [3]. The predominant mechanism of action of these two groups is believed to be direct G-protein activation, as pertussis toxin treatment inhibits both compound 48/80 [24] and substance P [25] induced secretion.

It has been shown *in vivo* that MC produced TNF is a critical factor in bacterial clearance [26]. Furthermore, MC express a number of Toll-like receptors (TLR), which enable them to directly respond to a plethora of bacterial and viral products, including Gram-negative lipopolysaccharide (LPS) (via TLR4 and 2), Gram-positive peptidoglycan (via TLR2), and double stranded RNA from viruses (via TLR3) [11]. Additionally, MC can indirectly respond to pathogen products using IgG and complement produced during infection. This is accomplished via Fcγ and complement receptors, respectively [11,27].

Finally, MC can be activated by a number of cytokines, chemokines and growth factors, including IL-1, IL-3, granulocyte/macrophage colony stimulating factor, IL-8 and SCF, and MIP-1α [3,28].

D. Mediators.

After activation, MC release a large range of biological effector molecules into their local environment. Indeed, it is this armamentarium of mediators which

allow MC to perform their important unique physiological roles. These mediators are either preformed and granule associated, synthesized from membrane phospholipid, or generated through *de novo* protein synthesis. They are released or produced upon appropriate stimulation [3]. An important characteristic of MC is the ability to selectively release a combination of the three types of mediators depending on the stimulus [3,11].

1. Granule associated.

Within MC granules, proteoglycans exist as both effector molecules as well as a storage units to facilitate packaging of other mediators, tryptase in particular [3,29]. Proteoglycans are proteins with long, charged carbohydrate side chains, which help in their structural role. Heparin and chondroitin sulfate are the proteoglycans associated with MC [3]. Notably, heparin is an anticoagulant when released from MC.

The most well-described preformed mediator is histamine. Histamine is produced in the Golgi by the decarboxylation of histidine, and is ionically linked with proteoglycans within the granule [3]. When MC degranulate, histamine dissociates from proteoglycan and acts on its target tissues, such as smooth muscle, through binding the specific H_1 and H_4 receptors [3].

Other notable components of MC granules are proteases. In the human subtypes MC_T and MC_{TC}, tryptase and chymase, respectively, are the best studied proteases [3]. They are associated with heparin in the granules [30], and have

multiple effects such as stimulating mucus secretion and the degradation of basement membrane components [31].

2. Lipid derived mediators.

In addition to rapidly releasing granule components, MC activation also leads to the prompt synthesis of potent mediators from arachidonic acid. PLA_2 converts phosphatidyl choline (PC) from both plasma and nuclear membranes to arachidonic acid, the critical substrate in the pathway. From this point, lipoxygenase enzymes (LO) catalyze the production of leukotrienes (LT) [32] and cyclooxygenases (COX) produce prostaglandins (PG) and thromboxanes [3]. Both LO and COX produced mediators have potent bronchoconstricting and chemoattractant properties [32,33]. Interestingly, human MC appear to preferentially produce LT or PG depending on their location, as lung MC preferentially produce LT, whereas skin MC preferentially make PG [34].

3. Cytokines and chemokines.

Lastly, MC are able to *de novo* synthesize a spectrum of cytokines (eg. IL-1, -3, -4, -5, -6, TNF, and IFN-γ) [3], and chemokines (eg. IL-8, I-309, MCP-1, MIP-1α, and RANTES) [35]. The transcription and translation of these factors takes several hours to produce significant amounts. As such, these are associated with "late-phase" responses of MC to stimuli, rather than the rapid effects of granule-associated and lipid-derived mediators. It should be noted that some cytokines, particularly TNF, have been shown to be preformed and stored in granules, allowing

for a rapid response in situations that involve TNF, such as host defence and allergic disease [36].

E. Mast Cells in Disease.

1. Allergic disease.

The development of allergic disease has long been associated with MC. Early degranulation causes Type I hypersensitivity. In asthma, this is characterized by bronchoconstriction, airway hypersensitivity, and airway swelling resulting from increased vascular permeability [3,37]. MC, though not the only cell type involved in this cascade, are considered to initiate asthma given their tissue residence in the lung, rapid allergen-IgE-mediated activation and important mediator secretion [3]. These early reactions are often coupled with a late phase responses (LPR) which occur hours following the initial response [37].

Early degranulation, which occurs after allergen-induced IgE crosslinking on the MC surface, results in the immediate release of the granule stored mediators as well as the production of lipid derived mediators (such as LT and PG) within minutes (<30 min) [3]. Histamine, LTC4, D4, and E4, and PDG2 induce bronchoconstriction, LTC4 and PGD2 increase microvascular permeability leading to airway swelling, and LTC4 induces mucus secretion [3,37].

The LPR, which occurs 4-24 hours post-allergen activation, is characterized by continuous swelling and inflammation resulting from the recruitment of basophils, eosinophils, neutrophils, lymphocytes, and monocytes/macrophages [38]. Interestingly, a number of MC mediators released in the early response contribute to

the LPR, including histamine, LTB4, PGD2, PAF and tryptase, that are chemoattractant for a number of the recruited cell types, and also upregulate adhesion molecule expression on endothelial cells to aid in their migration from the circulation [3]. Once recruited, these LPR effector leukocytes can be influenced by the array of cytokines that MC synthesized *de novo* (such as IL-5, known to be eosinophil activating) to foster and perpetuate the allergic response [37].

2. Parasitic infections.

In the gastrointestinal (GI) tract, MC have been implicated in host defence against parasitic infections [11]. Many of these infections result in the production of a large amount of parasite specific IgE by the host immune response, leading to activation of MC in the GI tract, and parasite killing [38]. Indeed, studies using the MC deficient W/W^{v} mouse have shown delayed parasite clearance when compared to normal littermates [39]. The clearance of these parasites is dependent on MC both releasing substances that directly harm the parasite [40], as well as generating an inflammatory response by recruiting effector cells such as eosinophils to the infection site [3].

3. Bacterial and viral infections.

Both allergic disease and parasitic clearance mediated by MC involve acquired immunity, in that a primary immune response must be generated by the classical pathway of antigen presentation, T cell activation, and B cell production of antigen specific IgE, to which MC can respond. A role for MC in innate defense against bacteria and viruses is emerging as well. This involves the ability of MC to directly respond to bacterial and viral products, in the absence of a primary immune

response [41]. MC display three key characteristics which make them ideal for innately responding to pathogens: 1) Their wide distribution in tissue, 2) Their location near blood vessels, in the skin, and in airways in the lung means MC can act as the first line of defense, and 3) Their ability to selectively produce and release different mediators depending on their stimulus (ie. allergen vs microbial products) [11,41].

As mentioned previously, MC express a number of TLR which are involved in their responses against bacteria and viruses. Human MC are reported to express TLR1, -2, -3, -4, -6, -7, -9 [11]. Furthermore, the expression of these receptors can be regulated by the MC microenvironment. For example, some MC do not express TLR4 until exposed to IL-4 or IFN-γ, when its expression is upregulated and the MC ability to respond to its ligand (LPS) is uncovered [42]. This suggests that the MC phenotype can be modified to respond to certain pathogens.

As previously discussed, the extensive array of mediators that MC can release is one of their defining features. In the setting of immune responses, MC produce a number of critical agents. For example, MC derived TNF is critical in the clearance of bacteria in *in vivo* models, to the point that administering TNF on its own to MC deficient W/W^{v} mice was sufficient to clearing the infection [26,43]. Recent work has also shown that MC produce antimicrobial peptides, such as cathelicidin [44]. Key to the MC effectiveness in infections is their ability to selectively release mediators depending on the stimulus. For example, in response to LPS, MC produce TNF, IL-1β, IL-6, GM-CSF, and chemokines, but do not degranulate [45], whereas in response to peptidoglycan they make IL-4, -5, -6, and

GM-CSF, as well as degranulating (resulting in edema and vascular permeability) [46]. Taken together, this evidence helps uncover how important MC are in the development of resistance to bacterial and viral infections.

4. Other disease processes.

MC have been implicated recently in a number of diseases and disease processes that were historically thought to be MC independent. These include, fibrotic diseases such as idiopathic pulmonary fibrosis (IPF), scleroderma, chronic graft-versus-host-disease (cGvHD), and liver fibrosis [47,48]. Alternatively, MC have been suggested to contribute to the processes of wound healing and repair [49], and to angiogenesis and the revascularization of damaged tissue [50].

Another group of diseases with a MC component are autoimmune conditions. These include rheumatoid arthritis, multiple sclerosis, chronic uticaria, pemphigus, and atherosclerosis [51]. Interestingly, a number of these diseases have a large T cell associated inflammatory component, in which MC are believed to play a role [51]. MC reside close to T cells in inflammatory settings, and have been shown induce T cell migration to inflammatory sites through release of chemotactic factors, as well as to activate T cells though antigen presentation and cytokine production (such as IL-4) [52]. In this way, MC are believed to contribute to the initiation of the inflammation characteristic of these diseases.

F. Experimental Mast Cell Models.

Due to their tissue-bound location, and the rarity of "mastocytosis", or mast cell leukemia, the study of human MC has been hindered by the absence of a good

cell model system. There are now two established cell lines, HMC-1 [53] and LAD-2 [54], both with benefits and drawbacks to their use.

HMC-1 display cytokine-independent growth, due to a functional mutation in c-kit (the SCF receptor), resulting in a brisk doubling time of 80 h; whereas, LAD-2 have a functional c-kit, therefore requiring the presence of SCF for culture, and have a doubling time of 2-3 wks [55]. The quick doubling time of HMC-1 allows for the easy generation of large amounts of cells to work with, though they do not appear to fully develop granules, and are considered "immature" [56]. On the other hand, the slow growth of LAD-2 allows for the development of MC corresponding to a late differentiation stage and expression of many MC markers, including chymase [54], but their culture requires SCF and generating sufficient cell numbers for investigation is inefficient.

Another major difference between these two cell lines is their expression of FcεRI; namely, LAD-2 show its expression whereas HMC-1 do not [55]. This is a critical difference, since human MC can be studied under physiological stimulation, such as IgE/anti-IgE activation using LAD-2, where models using HMC-1 are limited to non-physiological stimuli, such as Ca^{2+}-ionophore.

Since the demonstration that MC derive from CD34+ progenitor cells [57], the growth of experimental MC from CD34+ cells in human umbilical cord blood has been established, and the resulting culture termed cord blood MC (CBMC) [58]. These MC also require large amounts of SCF for their growth, and are labour intensive. However, CBMC express functional FcεRI and are a representative population for studying human MC, especially benefiting from the fact that they are

not tumorigenic in origin. Together, these cells allow for the effective study of human MC biology.

II. Intracellular Transport Systems

Within the cell, there are a number of organelles, such as the Golgi, nucleus, and endoplasmic reticulum, that generate products unique to each of them. In this way, they act as "factories" for the rest of the cell, which is the "consumer" [59]. The distribution and transport of these products depends on three different motor protein systems: myosin, kinesin, and dynein [59]. The majority of this section will focus on the function of the dynein transport system, but a review of all three is crucial to understanding their complete role.

A. Motor Protein Systems.

1. Kinesin.

Kinesin is a motor protein which contains an N-terminal motor domain, a long coiled-coil stalk, and a globular tail domain (Figure 1.1) [60]. The N-terminal (or head) domain binds directly to microtubule (MT) filaments making up the cytoskeleton of the cell [59]. Within cells, MT are polar, and are generally arranged with the 'minus' ends organized at a microtubule organizing center (MTOC) situated near the nucleus, and the 'plus' ends distributed outwards (Figure 1.2) [59]. Kinesin motors primarily move toward the 'plus' ends of MT [61], thus bringing their cargo to the cell periphery. Kinesin bind to their various cargo via their globular tail domain. The list of identified cargo for kinesin is extensive, and

includes proteins involved in cell signaling [62], vesicular proteins [63], viral proteins [64], and receptor associated proteins [65].

The head domains of kinesin have ATPase activity, and generate their motive force through the hydrolysis of ATP. This hydrolysis causes conformational changes which translate into a "hand-over-hand" mechanism of "walking" along MT based on the ATP occupancy of each ATPase domain (i.e. empty, ATP bound, or ADP bound) [66]. Therefore, kinesin are responsible for the outward distribution of a large number of unrelated proteins, and are key to the "long-distance" transport required by certain cellular systems.

2. Myosin.

There are a large number of myosin subfamilies, but the most heavily characterized and relevant to intracellular transport is myosin V [60], and is henceforth simply myosin. Myosin shares a large structural homology to kinesin, in that it contains a head domain responsible for ATP hydrolysis and force generation, a coiled coil stalk, and a globular tail responsible for binding it cargo (Figure 1.1) [59]. However, myosin travels along actin

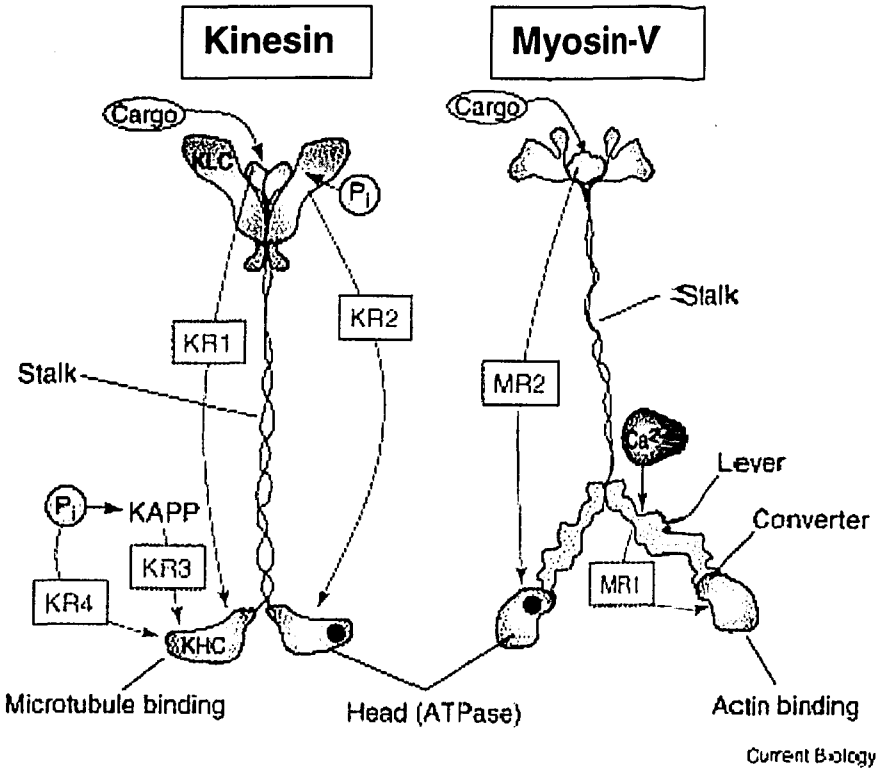

Fig 1.1 Diagram of kinesin and myosin motor proteins. Modified from [59]. Kinesin and myosin are homodimers of subunits which contain a N-terminal head domain which contains the ATPase portion, a long coiled-coil stalk, and a globular tail domain which is responsible for cargo binding. In addition, myosin contains a calcium (Ca^{2+}) binding lever region, and its activity can be regulated by intracellular Ca^{2+} levels. KR and MR indicate locations of kinesin and myosin regulation, respectively.

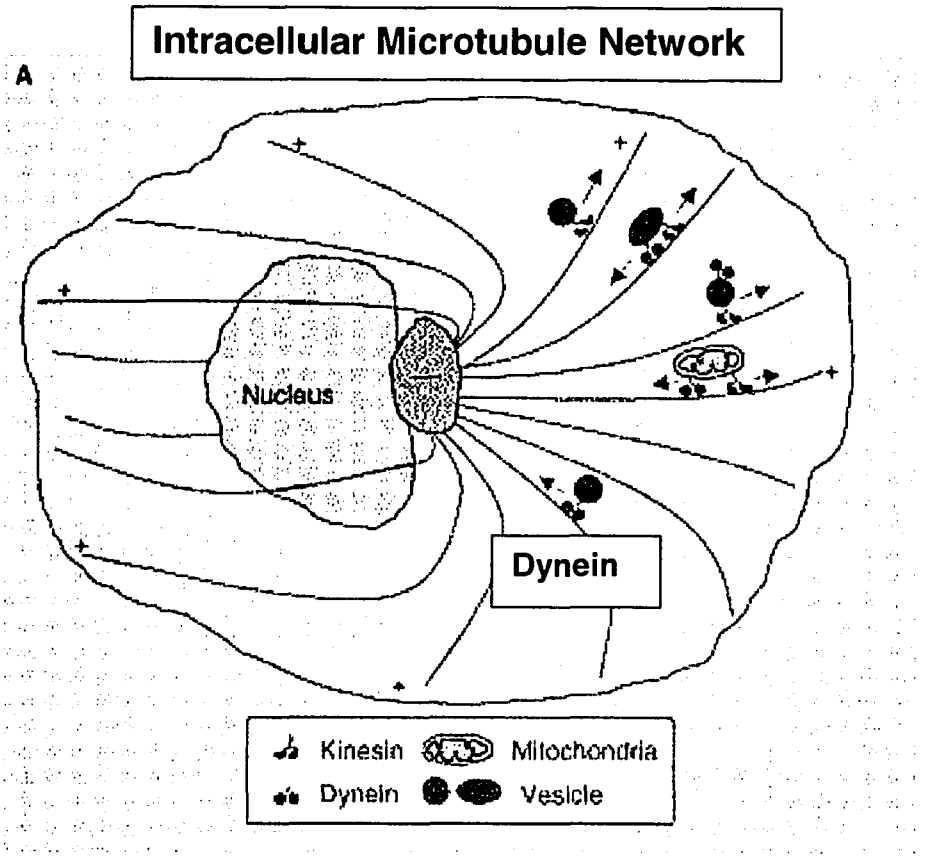

Fig. 1.2 Distribution and organization of microtubule (MT) network within cells. Modified from [59]. Negative ends are anchored at a microtubule organization center (MTOC) with the positive ends radiating out to the cell exterior. Both kinesin and dynein motors migrate along this network of MT, with their direction determined by the polarity. Kinesin moves towards the positive ends, while dynein move towards the negative ends

filaments, which are much shorter and more randomly oriented than MT [67]. This provides a more spread out network for a uniform distribution of cargo [68]. Interestingly, myosin is capable of binding to an additional light chain of the dynein family (LC8) [69], and the significance of this interaction will be discussed below.

3. Dynein.

Though dyneins are involved in coordinating intracellular transport along with kinesin and myosin, their structure and regulation are more complex.

a. Structure. The dynein family of proteins contains approximately 15 members, most of which are involved in ciliary and flagellar movement and named "axonemal" [70]. Two members are classified as "cytoplasmic" though only one of these, cytoplasmic dynein 1, is widely expressed and involved in intracellular transport [71]. Our discussion will focus on this form, and will be termed simply dynein.

Though dynein is a homodimer of identical heavy chains (HC), similar to kinesin and myosin, the ATPase containing head domains are more massive (~520 kDa) and complex (Figure 1.3) [59]. In addition to the two HC, dynein also contain two intermediate chains (IC), two light intermediate chains (LIC), and a variable number of light chains (LC) [72]. All three groups of accessory components have

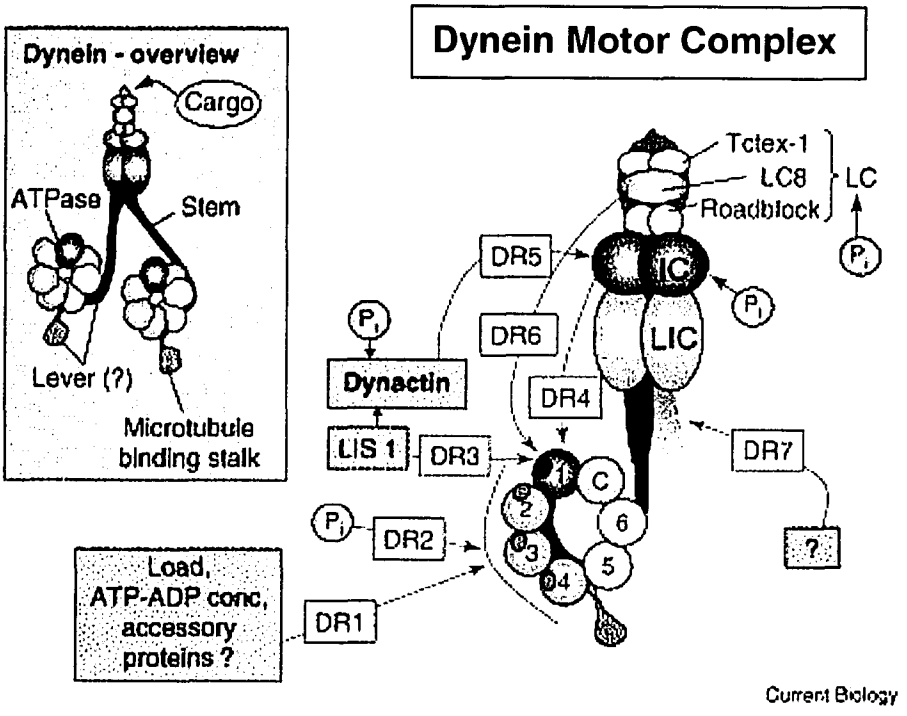

Fig. 1.3: Diagram showing structure of the dynein motor complex. Modified from [59]. Dynein is made up of a homodimer of heavy chains (HC) which contains the massive ~520 kDa ATPase unit, indicated here by the 6 ATPase associated with diverse cellular activity (AAA) subunits which facilitate the ATP hydrolysis. Additionally, the binding sites of the accessory light intermediate chains (LIC), intermediate chains (IC), and light chains (LC) are shown. DR indicates potential sites of dynein complex regulation.

cargo-binding capabilities. The total molecular weight of a complete dynein complex is around 1.2 MDa, almost 10 times larger than kinesin or myosin [73].

Dynein is an AAA protein, which stands for ATPase associated with diverse cellular activities [74]. This refers to the head region of the HC which contains many ATP binding sites (Figure 1.3) [59]. The head region has seven globular domains, of which six are AAA [75]. AAA1-4 can bind ATP while AAA5-6 cannot, and AAA1 is considered the primary ATP hydrolyzing unit [76,77]. Dynein interacts with its "tracks" through dedicated binding-stalks which project out from the AAA region of the head domain [78], further highlighting its difference from kinesin and myosin.

b. Function. Dynein motor complexes bind various cargo, and are responsible for their "retrograde" transport [70], meaning that they translocate along MT, but towards the negative ends (i.e. opposite of kinesin). Indeed, dynein is the major intracellular mode of transport towards the nucleus, and is necessary for a number of processes, including mitosis [79], neuronal transport [80], Golgi maintenance [81], and transport of numerous cargoes like mRNA, viruses, and endosomes [59].

Like kinesin and myosin, dynein are considered "processive" motors, meaning they are capable of multiple steps along MT before detaching, a property which makes them efficient transporters [59]. Dynein, however, take much larger "steps" than kinesin (15 nm vs 8 nm) [82,83], but the exact mechanism of dynein movement is unknown, as they do not appear to employ the "hand-over-hand" mechanism seen with kinesin and myosin [59]. The large size, compared to the two

other motor systems, and the presence of numerous accessory proteins indicate that dynein function is more highly regulated than its transport counterparts.

c. Accessory proteins. As previously mentioned, dynein complexes include a number of additional polypeptides, each with different roles in the regulation and function of the complete motor complex.

i. *Intermediate chain (IC)*: The IC in dynein (generally at a stoichiometry of two per complex [70]) is able to bind proteins which have important regulatory effects on the motor. Firstly, IC binds to the $p150^{Glued}$ subunit of the dynactin complex [84]. Dynactin can increase the processivity of dynein, demonstrated by increasing the run-length of dynein in *in vitro* assays [85]. Furthermore, dynein IC can interact with another protein, Lis1, which increases the stability of the dynein-dynactin complex, thus providing additional means of regulating dynein activity [86]. Finally, dynein IC can associate with β-catenin, a component of adherens junctions [87], suggesting that dynein interacts with these junctions, or is responsible for transporting β-catenin to the nucleus.

ii. *Light intermediate chain (LIC)*: LIC chains also appear to have cargo binding capabilities, albeit in limited amounts. LIC has been shown to bind to pericentrin [88], a centrosomal protein, facilitating its transport to the centrosome during mitosis.

iii. *Light chain (LC)*: There are three families of LC (LC8, Tctex, and roadblock/LC7) each capable of binding specific cargo. Indeed, LC are the primary method of cargo attachment to the dynein particle [89]. The latter two LC have a

fairly limited spectrum of identified binding partners, whereas LC8 has a long list of such associations [70].

The Tctex LC associates with rhodopsin, and is responsible for the correct apical sorting of this molecule [90,91]. Additionally, Tctex binds a number of receptors and ion channels, including the Trk neurotrophin receptor [92], CD155 [93], and CD5 [94], and the voltage-dependent anion-selective channel [95]. LC binding to receptors provides a unique way for receptors to interact with the cell interior after activation. Indeed the roadblock/LC7 LC highlights this phenomenon, as it is linked to the TGFβ receptor in epithelial cells, becomes phosphorylated upon receptor-ligand binding, and is required for downstream signaling events [96].

The most versatile of the LC is LC8. To date over 20 proteins [70] have been identified as LC8 binding partners. The diverse roles of these proteins include transcriptions factors, such as Kid-1 [97], the pro-apoptotic Bcl-2 protein, Bim [98], and the guanylate kinase domain-associated protein (GKAP) which is involved in signaling at post-synaptic membranes [99]. Furthermore, components of a number of viruses including herpes [100], lyssavirus [101], African swine fever virus [102], and the human immunodeficiency virus (HIV) [103] bind to LC8, and appear to "hijack" it and use it for transport to the nucleus.

LC8 also binds the p21-activated kinase 1 (Pak1) [104], an *in vivo* regulator of macropinocytosis, and the tumor suppressor p53 binding protein (53bp1) [105]. This interaction is critical to the DNA-damage induced translocation of p53 to the nucleus.

A unique aspect of LC8 is that it has also been characterized as an accessory protein to the myosin complex [69]. This provides an additional level of regulation to LC8 mediated transport, as it increases the transport potential and provides a mechanism to widely distribute a large number of proteins.

Interestingly, independent research in the field of nitric oxide (NO) identified LC8 as an inhibitor of neuronal nitric oxide synthase (nNOS) and termed the protein inhibitor of nNOS (PIN) [106]. PIN/LC8 was believed to bind to nNOS in its C-terminal region and disrupt its functional dimer, thus abrogating production of NO [106]. Subsequent work, however, has suggested PIN/LC8 is unable to affect nNOS production of NO, and rather has a role in determining the location of NO production via translocation of the dynein complex [107]. PIN/LC8 expression has been seen in human intestinal MC (unpublished data), and in HMC-1 and LAD-2 MC lines (chapter II).

B. Biochemistry of PIN/LC8 Interactions.

PIN/LC8 interactions with its various binding partners are dependent on a few key biochemical properties; the tertiary structure of PIN/LC8, the dimerization of PIN/LC8, and the presence of a conserved binding sequence in the target protein.

1. Leucine-rich repeat (LRR).

LRR are 20-29 amino-acid (aa) long sequences found in a number of unrelated proteins [108]. Generally, proteins containing these motifs are involved in protein-protein interactions [108]. They contain the consensus sequence LxxLxLxxN/CxL (where x can be any aa and L can also be V, I, or P) [108]. The

crystal structures of LRR have been solved, and each consists of a β-strand and an α-helix connected by loops [109]. PIN/LC8 contains six central LRR which are flanked by helical domains [108].

2. PIN/LC8 dimerization.

The crystal structure of PIN/LC8 binding a peptide of nNOS has been solved [110]. PIN/LC8 dimerizes in the presence of peptide *in vitro*, and thus potentially in the presence of complete cargo *in vivo*. The dimerization provides evidence for the model of PIN/LC8 interactions with the rest of the dynein complex, in that one monomer would bind the cargo, while the other would bind to the complex, either via IC, or directly to HC [110,111].

3. Conserved PIN/LC8 binding sequences.

To date there are two well characterized and conserved aa sequences which need to be present for successful PIN/LC8 binding. One consensus sequence is K/RxTQT (where x is any aa) [112], and the other is GIQVD [111]. Essentially, a beta-branched aa residue (such as threonine [T], or aspartate [D]) is located following a conserved glutamine (Q) residue [97]. There does not appear to be any particular pattern or protein type associated with either sequence. For instance, the K/RxTQT sequence is located in Bim, HC, Kid-1, and certain viral proteins, while GIQVD is found in nNOS and GKAP [113]. However, these sequences are not found in either IκBα or myosin [113], even though they have been identified as binding partners of PIN/LC8 using yeast two hybrid assays [69,114], suggesting that there are either other unidentified consensus sequences or that there are linker proteins to facilitate binding [97].

C. Motor Proteins in Disease.

As motor proteins are critical to the everyday workings within cells, it is not surprising that mutations or alterations in any of these proteins would lead to serious deleterious effects at the cellular level, but also the development of disease in the organism. Described below are some human diseases associated with various motor protein families.

1. Kinesin-linked diseases.

Mutation in the kinesin 1Bβ (KIF1Bβ) results in altered synaptic vesicle transport in neurons, causing Charcot-Marie-Tooth disease [115]. Symptoms include weakness, atrophy of distal muscles, arreflexia, and mild sensory loss.

Kinesin 5 is known to play a role in Alzheimer's disease, as amyloid precursor protein, which produces the pathogenic amyloid seen in the disease, is transported by KIF5 [116]. KIF5 has also been linked to spastic paraplegia, as a missense mutation in KIF5 is consistent with the development of the disease [117].

2. Myosin-linked diseases.

Myosin is associated with Grisselli syndrome, a rare autosomal-recessive disease characterized by pigment dilution in hair, severe immunological, and neurological symptoms [117]. It is clinically characterized by an altered transport profile of pigment-containing melanosomes, through either a mutation in myosin or other transport associated proteins which recruit myosin [118].

3. Dynein-linked diseases.

As mentioned previously, there are a large number of dynein subtypes, many which are involved in ciliary and flagellar movement. As such, a number of dynein associated diseases involve altered or deficient ciliary function in cell types where they are critical, such as the epithelium of airways. This is broadly termed immotile cilia syndrome, and results in chronic respiratory tract infection, sinusitis, and male infertility [117].

Cytoplasmic dynein, the form discussed extensively in this chapter, is associated with Lissencephaly, which is a developmental disease of the brain caused by disorganized cerebral cortex formation [119]. This disease is mainly caused by mutations in the gene encoding LIS1, which interacts with dynein [86], and may cause neural migration defects by affecting key developmental processes such as cell division, or nucleokinesis [117].

Additionally, NO has been shown to be a highly important biological mediator implicated in numerous physiological systems and disease states. As PIN/LC8 has been shown to bind to nNOS, it is likely that dynein plays an important role in a number of these NO-mediated events.

III. Nitric Oxide

A. Production.

Nitric oxide (NO) is produced by the nitric oxide synthase (NOS) family of enzymes. These enzymes catalyze the reaction of L-arginine to D- or L-citrulline, with N-hydroxyl-L-arginine occurring as an intermediate, and NO being produced

as a byproduct [120,121]. NOS enzymes require a number of cofactors to function including heme, tetrahydrobiopterin (BH_4), calmodulin, FAD, FMN, and NADPH (which is the electron donor), O_2, and the substrate L-arginine (Fig. 1.4)

There are three isoforms of NOS: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS), each isoform encoded by a separate gene [120]. nNOS and eNOS were named based on their initial tissue of discovery, while iNOS was discovered in an immunoactivated macrophage cell line [122]. NOS are only active when homodimers, and the individual subunits have molecular masses of 130 kDa (iNOS), 135 kDa (eNOS), and 155 kDa (nNOS) [120].

The NOS enzymes can be further classified as calcium-dependent and calcium-independent. All three isoforms require CaM to function, but nNOS and eNOS require above basal levels of intracellular calcium [$Ca^{2+}{}_i$], and are thus calcium-dependent [122]. Conversely, calcium-independent iNOS binds CaM at low [$Ca^{2+}{}_i$], and its activity is not regulated by intracellular calcium levels, unlike eNOS and nNOS [122]. Additionally, calcium-dependent NOS (nNOS and eNOS) produce lower amounts of NO than iNOS (pM vs nM).

B. Properties.

NO plays a key role in many physiological processes, including signal transduction and cytotoxicity [123]. This 30 Da free radical gas freely diffuses across membranes in cellular systems and directly interacts with a number of molecules.

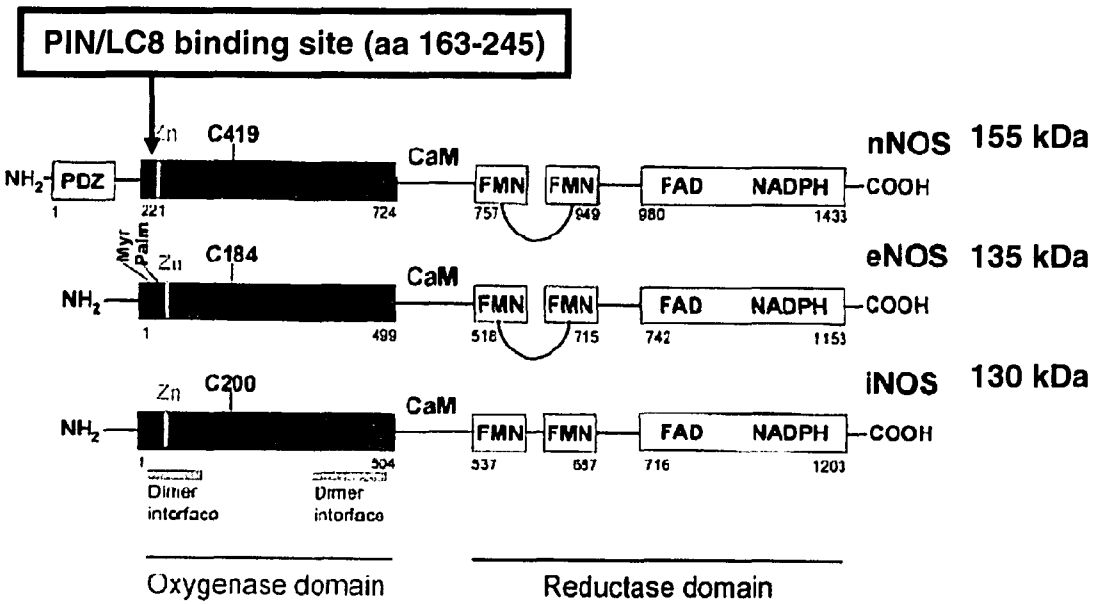

Fig. 1.4: Nitric oxide synthase (NOS) isoforms. Modified from [120]. The approximate molecular weight of neuronal NOS (nNOS) is 155 kDa, due to its extra N-terminal PDZ domain. Both inducible (iNOS) and endothelial (eNOS) are approximately 130 kDa. The oxygenase and reductase domains are the solid boxes, as indicated. Myristoylation (Myr) and palmitoylation (Palm) sites on eNOS are shown. Cofactor binding sites are indicated for: arginine (arg), BH_4, flavins (FMN, FAD), calmodulin (CaM) and nicotinamide-adenine-dinucleotide phosphate (NADPH).

The most well studied system which NO influences is the production of cGMP from the enzyme guanylate cyclase (GC). NO is able to interact with the iron heme of GC and increase formation of cGMP [124], which has a number of downstream effects including regulating vascular tone by relaxing smooth muscle, modulating synaptic transport, and inhibiting platelet aggregation [125].

An emerging property of NO is its ability to act as a signal transducing molecule, much in the same way as phosphate does in phosphorylation [126]. In addition to heme groups, NO is able to react with reduced sulfur (thiol) moieties, such as those in cysteine (Cys) residues. When this occurs, a *S*-nitrosothiol (SNO) is formed, and a protein containing an altered Cys residue is formed. To date, 115 different proteins are known targets for SNO formation [126] whose range of functions includes kinases, ion channels, transcription factors, structural proteins, proteases, and respiratory enzymes [127]. *S*-nitrosylation is considered reversible, in a similar fashion to phosphorylation.

NO, when in the form of peroxynitrate ($ONOO^-$), can also covalently modify proteins at tyrosine (Tyr) residues, through the addition of nitro (NO_2) groups, resulting in nitrotyrosines through a process called protein nitration [127]. Though initially used as a marker of pathological disease and oxidative stress, nitration [128] is now considered a physiological signal. Protein nitration was thought to be irreversible, but there is now evidence that these signals can be reversed by an enzyme termed denitrase [127].

NO is also able to indirectly affect biological processes through the formation of complex nitrogen oxides (NO_x) particularly when produced in large

amounts, such as in inflammatory conditions [129]. $ONOO^-$ and dinitrogen trioxide (N_2O_3) are two such species known to have potent effects on many important compounds including esters, guanosine, lipids, carbohydrates, and DNA [130].

C. Regulation of NO Production.

1. Protein-mediated regulation.

a. Heat shock protein-90 (HSP-90). HSP-90 is classically known as a chaperone protein, responsible for the correct folding and development of newly synthesized proteins [131]. Interestingly, HSP-90 is able to allosterically activate eNOS, increasing NO production up to three-fold [132]. This is believed to be due to a stabilizing effect of HSP-90 on the functional eNOS dimer [132]. Also, HSP-90 can act as a scaffold for other eNOS interacting proteins, such as the kinase Akt [133]. Recent work has identified a role of HSP-90 in nNOS activation as well. However, in this case, HSP-90 appears to facilitate binding of the heme group to nNOS, which is critical to NO production [134].

b. PDZ domain-containing proteins. PDZ domains facilitate protein-protein interactions, typically linking components of signaling pathways to large ternary complexes [135]. The N-terminus of nNOS contains a PDZ domain, unlike other isoforms of NOS, and is thus able to interact with a number of proteins. For instance, in neurons nNOS associates with postsynaptic density (PSD)-95 and PSD-93 proteins [136], which brings it in close proximity to the N-methyl-D-aspartate (NMDA) receptor, and is activated by the Ca^{2+} flux through this receptor when stimulated [137]. Furthermore, in the brain, another PDZ protein, C-terminal PDZ

ligand of nNOS (CAPON), competes with PSD-95 for nNOS binding and targets it to other locations [138].

c. Caveolin. Caveolae are cholesterol-rich invaginations in membranes known to sequester signaling molecules, and caveolin is a major protein component of these formations [139]. Caveolin-1 has been shown to bind eNOS, located in caveolae due to acylation at its myristoylation and palmitoylation sites, and inhibits its production of NO [135]. However, if intracellular Ca^{2+} levels rise, CaM binds to eNOS, dissociates it from caveolin, and promotes active enzyme formation [135]. This process is reversed when Ca^{2+} levels return to normal. Evidence for binding of caveolin to other NOS isoforms is weaker, though nNOS has been shown to bind caveolin-3 in skeletal muscle [140].

d. Receptors. A number of receptors are able to associate with NOS, and upon their binding of ligand and activation, either positively or negatively regulate NO production. The α_1-adrenergic receptors can associate with all three NOS isoforms [135]. Furthermore, the serotonin 5-HT2B receptor interacts with nNOS and eNOS and activates NO production [141]. Finally, the bradykinin B_2 receptor binds both eNOS and nNOS. Furthermore, a synthetic peptide representing the receptor binding sequence for the enzymes blocked electron transfer in nNOS, thus inhibiting NO synthesis [142].

e. Cofactors. The functions of all NOS isoforms are dependent on the presence of two key proteins: the enzymatic substrate arginine, and BH_4. Arginine is the nitrogen donating component of the NOS catalytic process, and its levels in the cell can be regulated by its extracellular uptake, protein catabolism, or synthesis

[143]. BH_4, largely produced by the enzyme GTP cyclohydrolase I [144], is also required for NOS function. Though to other enzymes it acts as an electron donor, its exact function in this system is unknown [144]. It has, however, been suggested that BH_4 aids in maintaining NOS in its active dimer form [145]. Both of these elements are regulatory to NOS by their presence or absence, and can influence the production of NO from all three NOS isoforms accordingly.

f. Other NOS binding proteins.

i. *eNOS traffic inducer (NOSTRIN).* NOSTRIN is able to bind to eNOS via its SH3 domain, and is able to redistribute eNOS within cells and inhibit NO production by this isoform [146].

ii. *eNOS interacting protein (NOSIP).* NOSIP appears to bind eNOS in a similar location as caveolin-1, but this interaction was not influenced by Ca^{2+}. NOSIP appears to uncouple eNOS from caveolae and block NO production, though the functional role of this interaction is unknown [147].

iii. *PIN/LC8.* As mentioned previously, PIN/LC8 is able to bind to nNOS at a specific region of its N-terminal region at the aa sequence GIQVD [113], though whether or not this interaction is designed to inhibit NO production or facilitate intracellular transport has yet to be determined.

2. Post-translational regulation.

a. Phosphorylation. Both nNOS and eNOS can be phosphorylated, though this modification alters the isoforms differently. eNOS is phosphorylated by the kinase Akt at its serine^{1179} residue which results in a 15-fold increase of NO production [148,149]. eNOS is also phosphorylated at threonine^{495}, likely by protein

kinase C (PKC), resulting in inhibition of NO production [150]. nNOS can also be phosphorylated at serine^{847}, resulting in a decrease in NO production [151].

b. Nitrosylation. As mentioned above, *S*-nitrosylation is the process of forming an SNO resulting in reversibly altered protein function. Recently, eNOS has been shown to be nitrosylated at cysteine^{99}, resulting in destabilization of active dimers and abrogated NO production [152]. Indeed, *in vitro* mutation of this cysteine to a residue unsusceptible to *S*-nitrosylation reversed this phenomenon [152]. This provides evidence for a new form of regulating NO production.

D. Nitric oxide and Mast Cells.

The expression of NOS and production of NO has been investigated in MC. Rat peritoneal MC (PMC) express eNOS under resting conditions, and the expression of iNOS can be induced after their treatment with the T_h1-type cytokine interferon-γ (IFN-γ) [153]. The expression of nNOS in PMC was not detected [153]. NOS expression varies amongst human MC populations. HMC-1, LAD-2, and skin MC all express eNOS, but only HMC-1 and skin MC express nNOS [154]. All human MC populations, however, do not appear to express iNOS [154]. Importantly, PMC, HMC-1, and LAD-2 have all been shown to produce endogenous NO, which results in the alteration of a number of MC functions [154, 155], and this effect can be enhanced by treatment with IFN-γ [155]. For instance, NO is able to inhibit release of numerous MC mediators, including histamine [156], and leukotrienes [154]. NO is also able to regulate other MC functions such as adhesion [158,159] and surface molecule expression [160].

IFN-γ also regulates a number of genes in addition to iNOS. Gene array analysis of HMC-1 has revealed that expression of several genes is increased, including the IFN-γ receptor and lymphotoxin β. With respect to NO production, expression of eNOS is also increased (unpublished data). Alternatively, of the many genes whose expression is inhibited by IFN-γ, PIN/LC8 is the most relevant considering MC production of NO, and suggests there may be a link between PIN/LC8 interactions with nNOS, and NO-dependent changes in MC function.

IV. Central hypothesis and objectives.

Because the spectrum of mediators that MC produce is so vast, and MC activating signals so numerous, the regulation of these responses is critical to the proper development of many physiological events. IFN-γ has an inhibitory role in many MC functions, in a NO-dependent fashion, and also decreases PIN/LC8 expression. Interestingly, the Th_2-type cytokines IL-4 and IL-10 tend to have opposing effects to IFN-γ on MC function, including NO production [160,161]. Additionally, PIN/LC8 has been shown to bind nNOS in numerous cell types, but this has not been studied in MC.

The central hypothesis is that IFN-γ regulates PIN/LC8 expression in MC in a manner opposite to IL-4 and IL-10. We further hypothesize that PIN/LC8 can bind a number of functionally relevant proteins in MC, and that one of these is nNOS.

Specific Objectives:

1. **To investigate expression, and cytokine-induced regulation, of PIN/LC8 mRNA and protein in human MC.** RT-PCR, real time PCR, and western blot analysis will be used to investigate expression of PIN/LC8 after treatment with the T_h1 cytokine IFN-γ , and T_h2 cytokines Il-4 and IL-10.
2. **To determine if PIN/LC8 is able to bind nNOS in human MC.** A recombinant GST-PIN/LC8 fusion protein will be used to perform pull-down assays for western blot analysis to detect the presence of nNOS.
3. **To interfere with PIN/LC8 protein-protein interactions and assess what effect this has on MC function.** A macromolecule delivery system will be used to introduce anti-PIN/LC8 antibodies into human MC culture and study the effects disrupting these interactions has on MC function.
4. **To identify additional binding partners of PIN/LC8 in MC.** In addition to nNOS, attempts will be made to identify other functionally relevant PIN/LC8 associated proteins in human MC. This will be accomplished through GST-PIN/LC8 pulldowns, followed by Q-TOF mass spectrometry analysis.

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Chapter 2

Regulation and Function of the Protein Inhibitor of Nitric Oxide Synthase (PIN)/Dynein Light Chain 8 (LC8) in Human Mast Cell Lines

Authors, S. D. McCauley, M. Gilchrist, and A. D. Befus. A version of this chapter has been submitted for publication. I contributed to this work by designing and carrying out the experiments and writing the manuscript.

Introduction:

Mast cells (MC) arise from CD34+ hematopoeitic progenitor cells in bone marrow [1] and as mature cells are often strategically located adjacent to blood vessels, nerves, and mucosal surfaces [2]. When activated through the high affinity IgE receptor (FcεRI), MC rapidly release preformed, granule-associated mediators; produce lipid derived mediators such as leukotrienes (LT) within minutes; and synthesize *de novo* numerous immunomodulatory and proinflammatory cytokines and chemokines over several hours [3-6]. Because of these unique characteristics, MC are involved in numerous biological processes, including innate immunity [2] and in Th-2 mediated inflammatory conditions, such as allergy and asthma [7].

The free radical nitric oxide (NO) is produced by the nitric oxide synthase (NOS) family of enzymes. Neuronal (nNOS) and endothelial (eNOS) NOS, are Ca^{2+}-dependent and release low levels of NO. The inducible (iNOS) isoform can be upregulated by various inflammatory factors, is Ca^{2+}-independent, and produces larger amounts of NO [8]. NO can inhibit MC functions associated with allergic reactions [9], and its production can be increased by exposure of MC to the Th-1 type cytokine, interferon-γ (IFN-γ) [9-12]. Conversely, Th-2 cytokines, such as interleukin (IL)-4 and IL-10 have been shown to activate MC, and as well can decrease production of NO in hematopoietic cell types [13,14]. Furthermore, various MC populations, including human skin MC (SMC) and human mast cell lines (HMC-1 and LAD-2) express eNOS and/or nNOS, but not iNOS [11,15] and, despite some controversy [15-17], NO production by MC has been demonstrated [11,12,17-19]. Interestingly, NO donors inhibit, while NOS inhibitors potentiate, the

production of cysteinyl LT from MC [11]. We postulated that MC express the protein inhibitor of NOS (PIN) and that PIN expression might be associated with cytokine regulation of NO production by MC.

Dyneins are multi-module protein complexes that act as molecular motors which provide the energy-dependent motive force for retrograde axonal movement along microtubules [20-23]. They are involved in vesicular transport, endosome and lysosome movement, ciliary and flagellar beating, and mitosis [20-22]. Cytoplasmic dynein complexes are made up of two heavy chains (HC), which generate movement through ATP-hydrolysis, and two intermediate chains (IC) which link the HC to its cargo by binding dynactin. Futhermore, two light intermediate chains (LIC) and several light chains (LC) are involved in regulatory functions, as well as binding dynein cargo [23].

One component of this complex, LC8, was independently identified as PIN [24]. Interestingly, immunoprecipitation, yeast two-hybrid and pepscan experiments have shown over 20 proteins capable of binding PIN/LC8 in addition to nNOS. Among others, these include Bim, a pro-apoptotic member of the Bcl-2 family [25], IκBα [26], the transcription factor Kid-1 [27], and a guanylate kinase domain-associated protein (GKAP) [28].

In this study we investigate the expression and regulation of PIN/LC8 in human MC lines HMC-1 and LAD-2, particularly when treated with cytokines known to influence NO production, namely IL-4, IL-10, and IFN-γ. Furthermore, we demonstrate the ability of PIN to bind nNOS in MC, and establish that anti-PIN

antibody enhances cysteinyl leukotriene (LT) production, albeit in an NO-independent manner.

Materials and Methods:

Cell Lines - The immature human MC line, HMC-1 (a gift from J.H. Butterfield, Rochester, MN) was cultured in Iscove's medium (Life Technologies, Grand Island, NY) with 10% fetal bovine serum (FBS), 2 mM glutamine, 40 U/ml penicillin/streptomycin and 1 mM thioglycerol. The mature, growth factor-dependent human MC line LAD-2 (obtained from Dr. A.S. Kirshenbaum and Dr. D.D. Metcalfe, NIH, Bethesda, MD) [29] was cultured in StemPro-34 medium (Life Technologies) supplemented with 100 ng/ml recombinant stem cell factor (rhSCF) (Peprotech, Rocky Hill, NY). All cells were maintained at 37°C in a humidified incubator at 5% CO_2.

Reverse-transcriptase Polymerase Chain Reaction (RT-PCR) - Total RNA was isolated from MC using TRIzol reagent (Invitrogen, Burlington, ON) according to the manufacturer's instructions. One microgram was converted to cDNA by the reverse transcription reaction (M.MLv reverse transcriptase, Invitrogen, Burlington, ON) in a volume of 20 µl. PCR amplification was carried out on a PTC-100 Thermal Cycler (MJ Research, Boston, MA). PIN/LC8 primers were designed to be intron-spanning based on published sequence data: PIN/LC8, sense 5'-TTCTCCACGGTAACCATGTG-3', antisense 5'-ACAACGTGGGCAGAAGTATG-3' (PCR product, 520 bp); β-actin, sense 5'-CCATGTACGTAGCCATCCA-3', antisense 5'-GATAGAGCCACCAATCCAC-

3' (PCR product 650 bp). PCR conditions were as follows: denaturing at 95°C for 45 s, annealing at 47° C for 45 s, and extension at 72 °C for 2 min. Products were run on a 2% agarose gel and stained with ethidium bromide (Sigma, St. Louis, MO). The amplified PCR products were subcloned into a pCR2.1 plasmid vector using the T/A cloning kit (Invitrogen, Carlsbad, CA). Plasmid DNA was isolated with the GenElute Plasmid-isolation kit (Sigma). Double-stranded DNA sequencing was performed using M13 forward and reverse primers. Sequencing was conducted using an ABI 373A automated sequencer (Applied Biosystems, Foster City, CA) by a dideoxy-chain termination method. RNA for positive controls was from human brain (Ambion, Houston, TX). Negative controls were RNA untreated with reverse transcriptase.

Real Time Polymerase Chain Reaction - Fluorophore-labeled LUX primers and unlabeled counterparts were supplied by Invitrogen. They were designed using the LUX Designer software (Invitrogen, www.invitrogen.com/lux); 5'6-carboxyfluorescein (FAM)-labeled PIN/LC8 5'-CAACATACCGCAATGTCCTTCTCTATG[fluorochrome]TG-3', Unlabeled PIN/LC8 5'-TGTCGGAAGAGATGCAACAGG-3'. Each 50 µl PCR reaction contained 1 µg RNA (isolated using RNeasy Mini Kit, Qiagen), 4.0 mM $MgSO_4$, 25 µl of 2X Reaction mix (Invitrogen) containing 400 µM of each dATP, dCTP, dGTP, and dUTP (for a final concentration of 200 µM each), 200 nM of each gene specific primer, 1 µl of Superscript III Reverse Transcriptase/Platinum *Taq* mix, and RNase/DNase free water to make volume up to 50 µl. Reactions were incubated at 50° C for 30 min (reverse transcriptase step), 95° C for 5 min, and then cycled 30x

using 95° C for 20 s, 55° C for 25 s, and 60° C for 30 s. Reactions were conducted in a spectrofluorometric thermal cycler (Rotor Gene RG-3000, Corbett Research). Fluorescence was monitored during every PCR cycle at the annealing and extension step. Fold increase/decrease was determined by comparing the critical fluorescence threshold values (Ct) for each sample in three independent experiments.

Western Blot - MC were incubated in six well plates at $2x10^6$ cells/well for 24 h in various experimental conditions. Cells were dissociated in cell lysis buffer (CLB) (20 mM Tris-HCL [pH 7.5], 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na_3VO_4, 1 μg/ml leupeptin) in the presence of 10 μM phenylmethylsulphonyl fluoride (PMSF) (Sigma). The total protein content was determined by the Bradford assay (BioRad). Protein samples (30 μg) were mixed with 3x SDS Sample Buffer (New England Biolabs) and separated on a 10% SDS-PAGE gel, transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA), and blocked overnight in 5% milk (Biorad) at 4°C. The membrane was incubated with primary antibodies for 1 h at room temperature. Primary antibody dilution: monoclonal PIN/LC8 (1/1000), monoclonal nNOS (1/1000), monoclonal eNOS (1/1000) (all from BD Transduction Labs, Lexington, KY), polyclonal actin (1/2000) (Santa Cruz Biotech, Santa Cruz, CA). The secondary antibodies, a 1/5000 HRP conjugated goat anti-mouse IgG (Serotec, Raleigh NC) and 1/10000 HRP conjugated goat anti-rabbit IgG (Serotec) were added and incubated for 1 h at room temperature. Blots were washed after primary and secondary antibodies in tris buffered saline (TBS) [pH 7.6] containing 0.1% Tween-20. Specificity of anti-PIN/LC8 confirmed by peptide blocker (Santa

Cruz). The use of nNOS and eNOS antibodies has been published previously [11]. Labelling was detected by chemiluminescence by addition of ECL Western Blotting Detection System (Amersham, Buckinghamshire, UK). The bands were scanned and quantified using an AlphaImager 2200 (Alpha Innotech, San Leandro, CA).

Purification of recombinant GST-PIN protein - *E. coli* BL21 containing pGEX-4T-1 plasmids encoding glutathione-S-transferase (GST) and GST-PIN were a kind gift from Dr. Tom Magee and Dr. Nestor F. Gonzales-Cadavid, UCLA, Los Angeles, CA. GST and GST-PIN were isolated as described previously [30,31]. Briefly, bacteria were grown with shaking at 37°C to an optical density of 0.6 at 600 nm in 250 ml of Luria-Bertani (LB) medium. To induce recombinant protein production, isopropyl-β-$_D$-thiogalactopyranoside (IPTG) (Amersham) was added to a final concentration of 1 mM, and bacteria growth was continued at 37° C for 2 h. Bacteria were harvested and washed twice in PBS, and the pellet resuspended in GST buffer (PBS + 1% Triton X-100). Bacteria were lysed by sonification at 0° C. GST-PIN and GST were purified from total lysate by diluting 1 ml of lysate into 4 ml of GST buffer, adding a 50% slurry of glutathione-sepharose 4B (Amersham) in GST-buffer, and rocking for 1 h at 4° C. The bound GST-PIN and GST proteins were eluted for 1 h using 500 µl of glutathione elution buffer (10 mM glutathione in 50 mM Tris [pH 8.0]), and analyzed for purity by SDS-PAGE followed by silver staining.

GST-PIN Pulldowns - MC ($1x10^7$ cells) were dissociated in 200 µl radioimmunoprecipitation assay (RIPA) buffer (phosphate buffered saline [PBS], 1% NP-40) on ice for 30 min in the presence of PMSF, centrifuged at 8000 xg for

10 min, and supernatants collected ("lysate" fraction). 50 μg of GST and GST-PIN proteins were incubated with 100 μL lysate, rocking overnight at 4° C. Glutathione-sepharose 4B (300 μl; Amersham), was then added and rocked for 2 h at 4° C. Samples were centrifuged at 300 xg for 5 min and the supernatant was discarded. They were then washed extensively with HNTG buffer (20 mM HEPES [pH 7.4], 500 mM NaCl, 10% glycerol, and 0.1% Triton X-100). GST proteins were then eluted with 200 μl glutathione elution buffer for 2 h at 4° C, and analyzed by western blot. Prebinding of polyclonal anti-PIN/LC8 (Santa Cruz Biotech) was performed by adding 2 μg anti-PIN/LC8 to 50 μg of GST-PIN for 30 min at 4° C prior to adding to lysate.

Antibody Dialyzation - Both the polyclonal anti-PIN/LC8 (Santa Cruz), whose specificity for PIN/LC8 has been confirmed by peptide blocking experiments (Santa Cruz), and purified polyclonal rabbit IgG (Serotec) used with Chariot were removed from their original solutions containing sodium azide through filtration using a Microcon YM-3 centrifugal filter device (Millipore). Two hundred μl of either anti-PIN/LC8 or rabbit IgG were added to the filter, then centrifuged for 30 min at 13,000 xg until all the liquid had flowed through the filter. Antibody was recovered by inverting the filter and centrifuging for 3 min at 1000 xg. Filtrate was diluted in sterile PBS, and protein content was determined using the Bradford assay (Biorad).

Chariot-mediated Antibody Delivery - HMC-1 ($1x10^6$ cells) were reconstituted in 1 ml of complete medium and allowed to adhere to 12 well plates for 1 h. In the meantime, 100 μl of reconstituted Chariot (ActiveMotif, Carlsbad, CA) was incubated with 100 μl of PBS containing the appropriate amount of antibody (either

polyclonal anti-PIN/LC8, Santa Cruz, or rabbit IgG control, Serotec) and allowed to form the Chariot-antibody complex for 30 min at room temperature. The complete medium was aspirated from the adherent cells, and 200 µl Chariot-antibody complex (and appropriate controls: cells treated with PBS alone, and cells treated with Chariot in the absence of antibody) were added to the cells, followed by 150 µl of serum-free media, and allowed to enter the cells for 1 h at 37° C. One ml of complete medium was then added and the cells were further incubated for 2 h at 37° C. The cells were then stimulated for 30 min with 2 µM Ca^{2+}-ionophore (A23187) and supernatants were collected for analysis. The NOS inhibitor N^{G}-nitro-$_{L}$-arginine methyl ester (L-NAME) treated samples were treated identically, except increasing concentrations (100, 500, and 1000 µM) of L-NAME were added 30 min prior to stimulation with A23187.

Enzyme-linked immunosorbent Assay (ELISA) for Cysteinyl Leukotrienes - After the appropriate treatment, HMC-1 cells were stimulated with A23187 (2 µM) for 30 min at 37° C. Culture supernatants were collected and cysteinyl LT (LTC_4, D_4, E_4, C_5, D_5, E_5) were quantified using an Enzyme Immuno Assay (EIA, Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's protocols.

Statistical Analysis – Data were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test for post-hoc comparisons except for real-time PCR which was analyzed using the non-parametric Kruskal-Wallace test.

Results:

Expression of PIN/LC8 mRNA in HMC-1 and LAD-2 cells

To determine if PIN/LC8 mRNA was expressed in these two cell types, RNA was extracted from untreated HMC-1 and LAD-2, and RT-PCR was performed. It was identified that these cells express PIN/LC8 mRNA (Fig. 2.1, A and B). The PCR product was cloned and sequenced as described in "Methods and Materials", and the identity of the product confirmed as PIN/LC8.

Cytokine induced regulation of PIN/LC8 mRNA expression in MC lines

To determine if the T_h1 cytokine IFN-γ and the T_h2 cytokines IL-4 and IL-10 affected PIN/LC8 expression, the relatively immature HMC-1 and more mature LAD-2 cells were incubated overnight in the presence of these cytokines before RNA was extracted. RT- and real-time PCR were then performed with PIN/LC8 specific primers and compared with appropriate controls to determine differences in the amount of PIN/LC8 mRNA. IFN-γ treatment down regulated PIN/LC8 mRNA in both HMC-1 and LAD-2 cells (Fig. 2.2, A and B); quantitative studies showed a -1.9 ± 0.4 fold (HMC-1) and a -1.5 ± 0.3 fold (LAD-2) decrease. By contrast, IL-4 increased the PIN/LC8 mRNA in both cell types (Fig. 2.2, A and B), but to a lesser extent in LAD-2 (+1.6 ± 0.1 fold) than the significant increase in HMC-1 (+7.6 ± 0.5 fold, $P<0.05$) (Table 2.1). The real-time PCR studies confirm the RT PCR results and emphasize the differences in sensitivity of the two MC types to treatment with cytokines. IL-10 treatment had less marked effects than IL-4 on

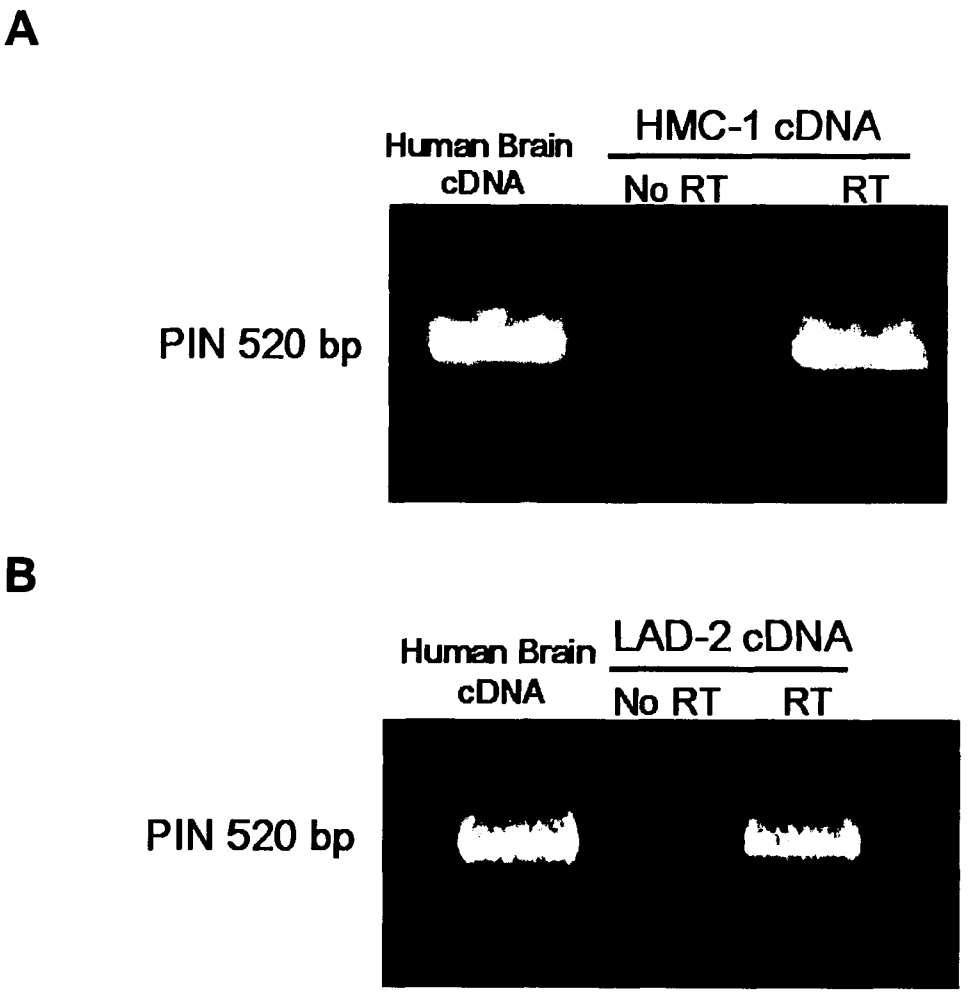
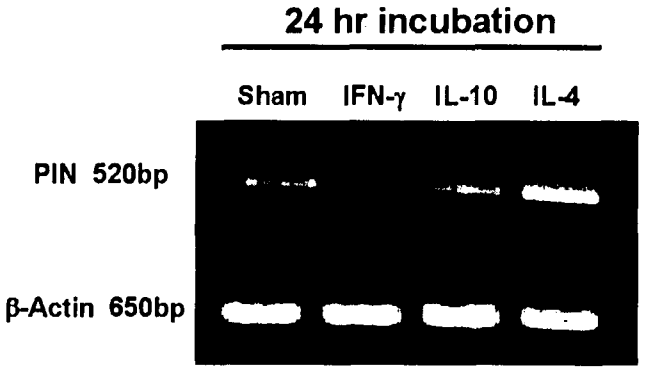


Fig 2.1: Expression of PIN/LC8 mRNA in human mast cell populations. RT-PCR analysis of unteated HMC-1 (A) and LAD-2 (B) show the expression of PIN/LC8 mRNA (Lane 3). Negative controls for each group were RNA which had not undergone reverse transcription (No RT, Lane 2). Positive controls for the PCR reactions were human brain cDNA samples (Lane 1). PCR was run as described in "Materials and Methods" for 30 cycles prior to visualization. Size of products indicated in base pairs (bp).

A



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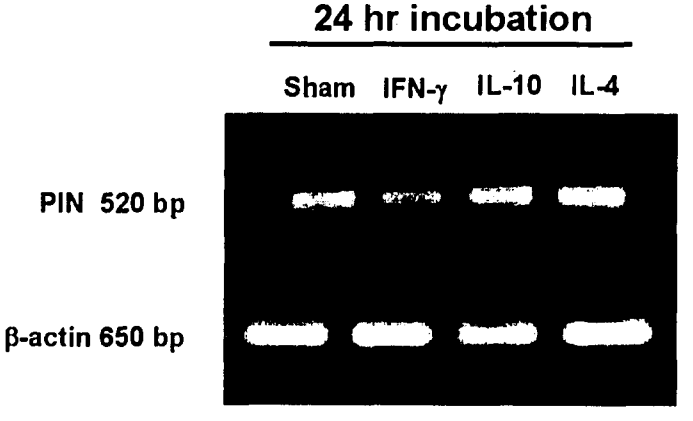

Fig 2.2: Regulation of PIN/LC8 mRNA in human mast cell populations. RT-PCR analysis shows the expression of PIN and β-Actin mRNA in HMC-1 (A) and LAD-2 (B) when untreated (Lane 1) or treated with IFN-γ (100 ng/mL) (Lane 2), IL-10 (50 ng/mL) (Lane 3), or IL-4 (100 ng/mL) (Lane 4) for 24 h at 37° C. PCR was run as described in "Materials and Methods" for 30 cycles prior to visualization. Size of products indicated in base pairs (bp). Results representative of five independent RNA isolations.

the amount of PIN/LC8 mRNA in both cell types, but increased PIN/LC8 expression by +1.5 ± 0.5 fold in HMC-1 (Fig 2.2; Table 2.1).

Cytokine induced regulation of PIN/LC8 protein expression in MC line lysates

Given that expression of PIN/LC8 mRNA was influenced by cytokine treatment, PIN/LC8 protein levels in HMC-1 and LAD-2 lysates were investigated following the same treatments. In both cell types, IL-10 treatment had limited effects on PIN/LC8 protein found in lysates, although expression was increased approximately 30% in LAD-2 (Fig 2.3B; n.s., $P>0.05$). IFN-γ decreased PIN/LC8 protein in HMC-1 by ~50% (Fig 2.3A; $P<0.001$), but had a negligible effect in LAD-2 (Fig 2.3B; n.s., $P>0.05$). IL-4 treatment produced contrasting results on levels of PIN/LC8 protein in the two cell types. In HMC-1, IL-4 treatment reduced PIN/LC8 protein in cell lysates ~ 50% ($P<0.001$) whereas LAD-2 treatment with IL-4 more than doubled PIN/LC8 protein in the cell lysate ($P<0.001$) (Fig 2.3, A and B).

Ability of PIN/LC8 to bind nNOS in HMC-1

Previous work established that PIN/LC8 can associate with nNOS in human embryonic kidney (HEK) 293 cells and disrupt production of NO [24]. Thus, we hypothesized that PIN/LC8 would bind nNOS in MC. To identify if PIN/LC8 could physically associate with nNOS in HMC-1 in vitro, we used recombinant GST-PIN in pull-down experiments (HMC-1 were used exclusively in these experiments as we had previously found that LAD-2 do not express nNOS) [11]. GST-PIN and control

Table 2.1: Fold increase or decrease of PIN/LC8 mRNA in HMC-1 and LAD-2 cells after cytokine treatment as determined by real time PCR[1].

Treatment	Fold Increase/Decrease	
	HMC-1	LAD-2
IFN-γ	-1.9 ± 0.4	-1.5 ± 0.3
IL-4	+7.6 ± 0.5*	+1.6 ± 0.1
IL-10	+1.5 ± 0.5	+0.8 ± 0.3

[1]Results shown as mean fold increase/decrease ± SEM for three independent experiments.
*P<0.05

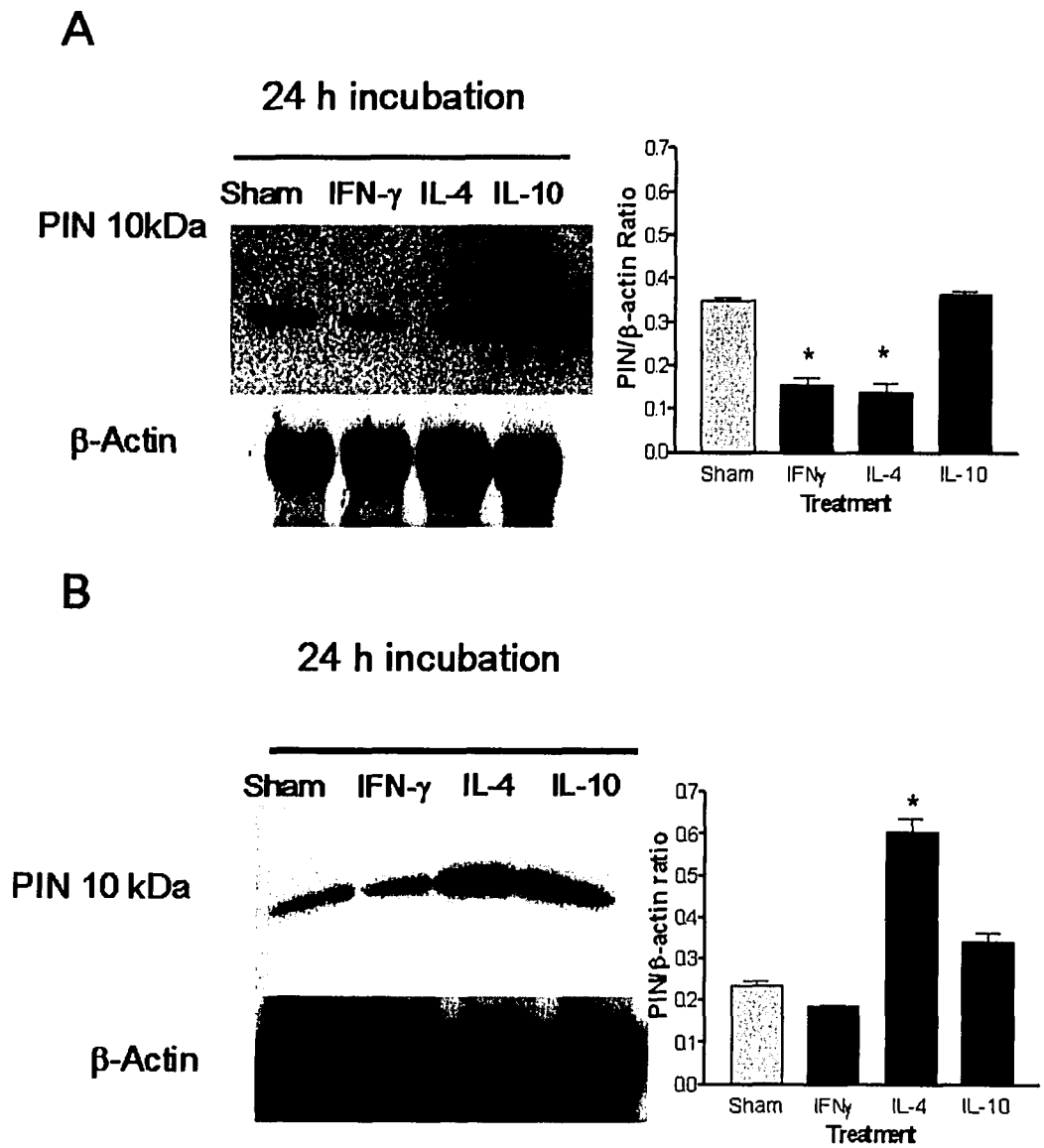

Fig 2.3: Western Blot analysis of PIN/LC8 protein regulation in human mast cells. Mast cell lysates were generated as described in "Materials and Methods", and equal amounts of protein were loaded (30 μg) for untreated samples (Lane1) and samples treated with IFN-γ (100ng/mL) (Lane 2), IL-4 (100 ng/mL) (Lane 3), or IL-10 (50 ng/mL) (Lane 4) for 24 h at 37° C of HMC-1 (A) and LAD-2 (B) cells. Blots were stripped and reprobed for β-actin for loading control. Densitometric analysis, shown as PIN/β-actin ratio expressed in arbitrary units (mean ± SEM) is from five independent experiments. *P<0.001

GST proteins were exposed to HMC-1 lysate, and the resulting pull-down fractions were analyzed by western blot. nNOS was identified in GST-PIN samples (Fig 2.4A, Lane 3) but not in the GST control samples (Fig 2.4A, Lane 2). By contrast, eNOS was not detected in any pulldown sample (Fig 2.4B), even after treatment of MC with IFN-γ, which increases eNOS expression (our unpublished data) and IL-4, known to activate MC, particularly their production of LT [14,32]. This is not surprising, as only nNOS contains the consensus sequence for PIN/LC8 binding [8,27,33]. Furthermore, pre-incubating the GST-PIN with an antibody specific to PIN/LC8 prior to exposing it to the cell lysate abolished binding to nNOS (Fig 2.4B, Lane 4). Thus, we demonstrated that PIN/LC8 binds nNOS, but not eNOS, in HMC-1.

Effects of intracellular delivery of anti PIN/LC8 antibody on leukotriene release

Because PIN/LC8 can bind nNOS in HMC-1, we used anti-PIN/LC8 antibodies to investigate the potential role of PIN/LC8 in LT production. Our rationale was based on our previous results showing that exogenous NO donors inhibit LT production [11], and results from this study showing that PIN/LC8 binds nNOS. We predicted that blocking PIN/LC8 interaction with nNOS would mimic these inhibitory effects on LT production because there would be more unbound nNOS and NO production than in untreated conditions.

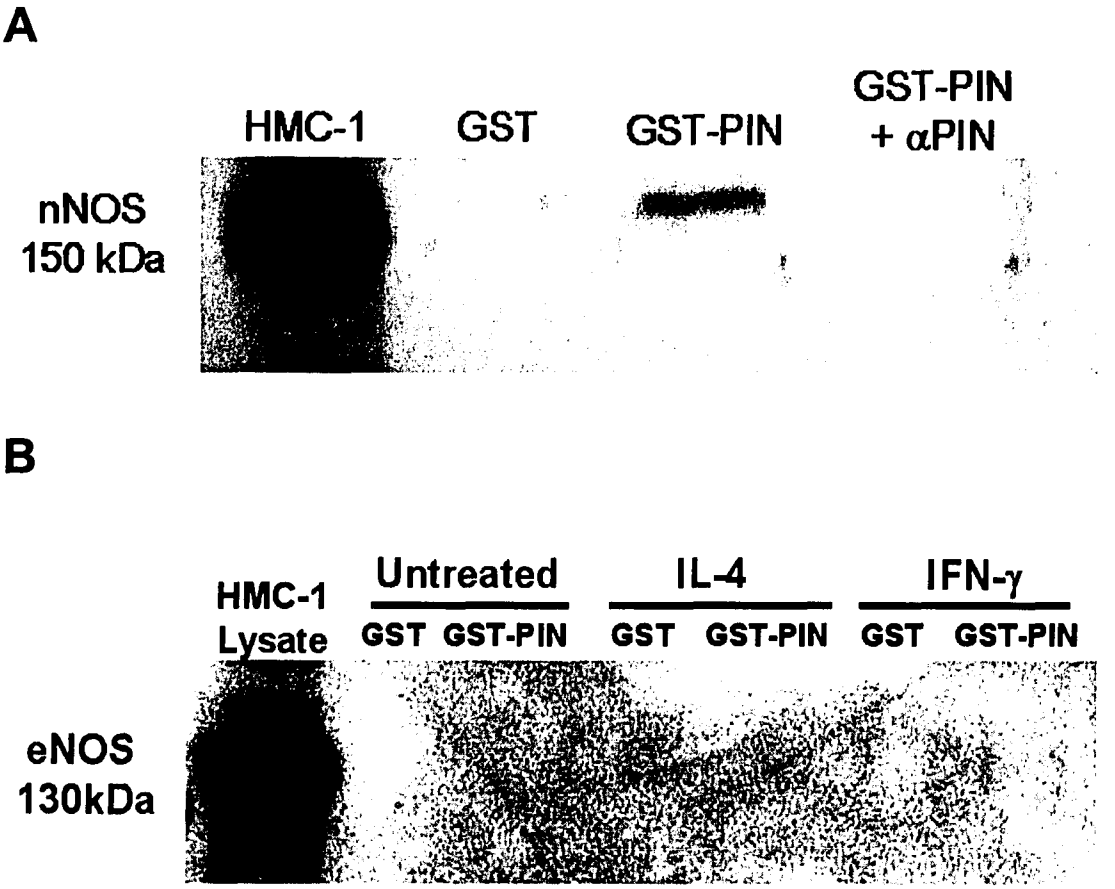

Fig 2.4: Binding of recombinant GST-PIN to nNOS, but not eNOS, in HMC-1 lysate: (A) 50 μg of GST-PIN or GST alone was incubated with HMC-1 lysate for 18 h at 4° C while rotating. The GST-PIN and GST proteins were then bound to glutathione-sepharose 4B, washed, and eluted as described in "Materials and Methods". Equal amounts of eluant (40 μL) from GST (Lane 2) and GST-PIN (Lane 3) were separated by SDS-PAGE under reducing conditions and subject to western blot analysis for nNOS. Anti-PIN prebinding (Lane 4) was performed by incubating the GST-PIN with 2 μg of polyclonal antibody specific for PIN for 30 min at 4° C prior to adding to HMC-1 lysate. HMC-1 lysate (Lane 1) run concurrently as positive control for the presence of nNOS. (B) Analysis of GST-PIN binding to eNOS performed as above, except for HMC-1 treated for 18 h at 37° C with IL-4 (100 ng/mL) (Lanes 4 and 5) and IFN-γ (100 ng/mL) (Lanes 6 and 7) were included in nNOS western blot analysis in addition to untreated cells (Lanes 2 and 3). HMC-1 lysate (Lane 1) run concurrently as positive control for presence of eNOS. Western blots are representative of four independent pulldown experiments.

We introduced PIN/LC8 specific antibodies into HMC-1 using a macromolecular delivery system, Chariot [34]. After introducing increasing concentrations of anti-PIN/LC8 into HMC-1 and stimulating for 30 min with 2 μM A23187, we saw a statistically significant stimulus and dose-dependent increase, rather than the predicted decrease, of cysteinyl LT in culture supernatants (Fig 2.5A, Columns 4-6) when compared to untreated (Fig 2.5A, Columns 2 and 3) and unstimulated (Fig 2.5A, Columns 1 and 7) controls. Furthermore, introduction of non-specific IgG resulted in no significant alterations in LT production, even at twice the concentration of antibody. Thus, specifically interfering with PIN/LC8 with a polyclonal antibody significantly enhanced LT production by HMC-1.

L-NAME effects on Anti-PIN/LC8 induces leukotriene production

To determine if the anti-PIN/LC8 antibody enhancement of LT production involved NO, antibody-treated (1 μg) HMC-1 were given increasing concentrations of the NOS inhibitor L-NAME prior to A23187 stimulation (2 μM). However, this pharmacological inhibition of NOS had no significant effect on LT production when compared with cells untreated with L-NAME (Fig 2.6). Thus, NO has no obvious role in the anti-PIN/LC8 induced increase of LT production in HMC-1.

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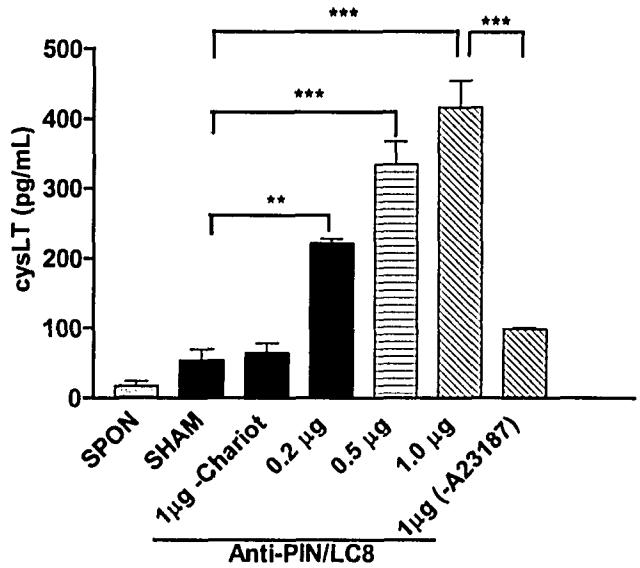

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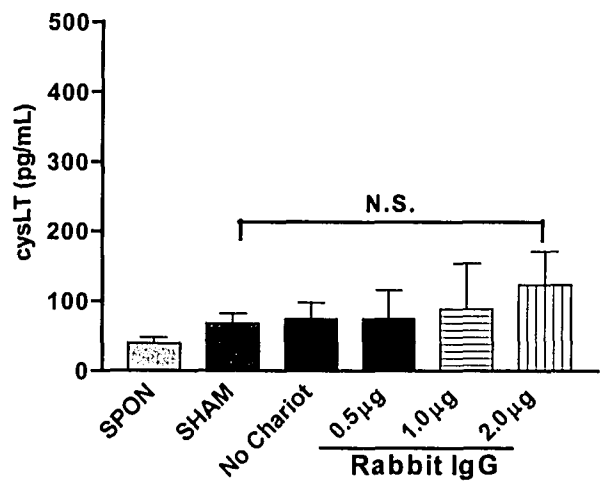

Fig 2.5: Anti-PIN/LC8 induced potentiation of leukotriene production by HMC-1: The introduction of increasing amounts of anti-PIN IgG (0.2, 0.5, and 1.0 µg) (A) and control IgG (0.5, 1.0, and 2.0 µg) (B) was performed using the Chariot tool, as described in "Materials and Methods". Culture supernatants were collected for quantification of cysteinyl LT produced after stimulation of cells with A23187 (2 µM) for 30 min. In (A), the antibody treated groups (Columns 4-6) showed significant increases in LT production when compared with Sham (untreated) (Column 2), Chariot-free (Column 3), and Ca^{2+}-ionophore free (unstimulated) (Column 7) samples. The antibody treated groups in (B) (Columns 4-6) showed no significant increases in LT production when compared to Sham and Chariot-free samples (Columns 2 and 3). Results are expressed as mean ± SEM for four independent experiments. *P<0.01, **P<0.001.

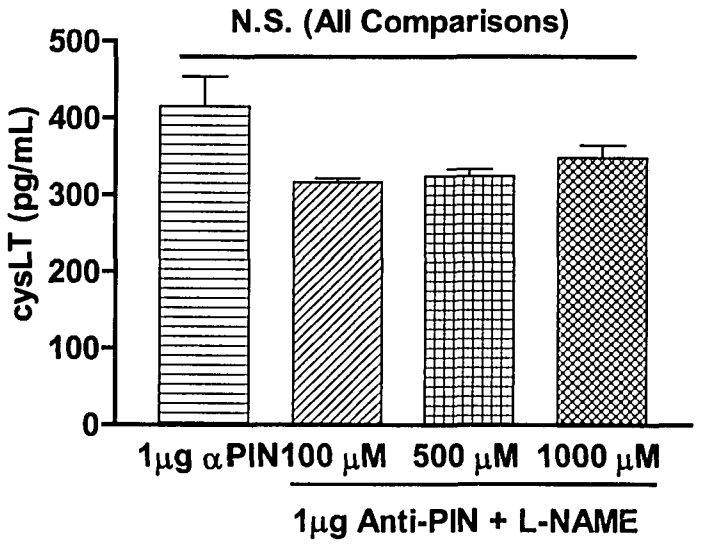

Fig 2.6: Effect of NOS inhibition on Anti-PIN/LC8 enhanced leukotriene production: Anti-PIN IgG (1 μg) was introduced to HMC-1 using Chariot, and cells were stimulated with A23187 (2 μM) prior to supernatant collection and analysis as described in "Materials and Methods" and Figure 2.5. Additionally, samples were treated with increasing concentrations of L-NAME (100, 500, 1000 μM) (Columns 2-4) for 30 min prior to stimulation with A23187. The L-NAME treated samples showed no significant difference in LT production when compared with non L-NAME treated samples that contained the same amount of anti-PIN. Results expressed as mean ± SEM for three independent experiments.

Discussion:

To our knowledge, this is the first report showing the expression and potential functionality of PIN/LC8 in human MC. Both HMC-1 and LAD-2 cell lines express PIN/LC8, and exposing the cells to T_h1 and T_h2-type cytokines influenced this expression. Expression of mRNA in both cell types is similarly regulated. IFN-γ causes a down-regulation of PIN/LC8, while IL-4 increases it. IL-10 appears to play a lesser role in the regulation of PIN/LC8 expression. The extent of cytokine-induced regulation of PIN/LC8 expression, however, varies between the two cell lines. HMC-1 are more "sensitive" to these cytokines than LAD-2, in that the fold increase and decrease of gene expression is greater in HMC-1 than LAD-2. LAD-2 cells are a more mature phenotype than HMC-1, which may help to explain these differences [29]. HMC-1 have a mutation in the stem cell factor (SCF) receptor, c-kit, and thus are growth factor independent, whereas LAD-2 are SCF-dependent, and their transforming mutation has yet to be identified [29]. To this end, LAD-2 have a doubling time of 2 wk, and have many characteristics of terminally differentiated MC [29,35], as compared with HMC-1 whose doubling time is approximately 80 h [36].

PIN/LC8 protein regulation in the two cell types also showed differences. HMC-1 were more sensitive to IFN-γ, with a notable decrease of PIN protein in lysates, whereas LAD-2 showed little if any response. IL-10 had a minimal effect on PIN/LC8 protein expression. IL-4 treatment gave contrasting results. LAD-2 displayed a ~ 2 fold increase in protein, which is consistent with the increase in

mRNA, whereas HMC-1 showed a ~50% depletion in PIN/LC8 protein after treatment. The relative maturity of HMC-1 and LAD-2 may account for these differences. Studies have shown that MC populations respond differentially to various stimuli, particularly IL-4, based on their maturity level [35,37]. It is possible that the differences seen may not result from changes in protein synthesis or catabolism, but from alterations in intracellular localization of PIN/LC8. Dyneins transport their cargo towards the minus end of microtubules, which are generally anchored to the nucleus [21,22], but our procedure for MC lysis enriched the cytoplasmic, rather than the nuclear, fraction. Therefore, in HMC-1, IL-4 could increase dynein movement to the nucleus and surrounding organelles which could account for the lack of protein seen in western blots. However, when protein was extracted from HMC-1 nuclei following IL-4 treatment, western blots for PIN/LC8 were negative, suggesting that this is not the dominant mechanism to explain the differences in IL-4 mediated PIN/LC8 expression between HMC-1 and LAD-2 (data not shown).

To begin to understand what role PIN/LC8 plays in MC regulation, we investigated if PIN/LC8 was able to associate with NOS. There are two consensus sequences described which facilitate PIN/LC8 binding, both of which contain a critical glutamine (Q) residue [27,33]. The sequence found in nNOS is GIQVD, and it is not found in eNOS [38] (Figure 1.4). Using recombinant GST-PIN, we demonstrated that PIN/LC8 and nNOS, but not eNOS, associate in HMC-1. LAD-2 do not appear to express nNOS [11], and this observation was supported by the lack of association between nNOS and PIN in LAD-2 lysates (data not shown). Taken

together, this evidence suggested that PIN/LC8 might regulate NO-mediated effects in HMC-1, as well as human MC *in vivo* known to express nNOS, such as nasal mucosa and ileocaecal tissue derived MC [39].

The ability of NO to regulate MC function is becoming widely accepted [9,10,40], although controversy has developed regarding the ability of MC to produce NO. Swindle *et al* [15] found that MC are not able to produce NO on their own, and that the NO effects are caused by exogenous NO produced by surrounding cell types. These findings contrast with ours where we have identified using a number of techniques that MC produce NOS isoforms and NO [11,17-19]. However, recently we have identified that the contrasting results likely reflect strain differences in the rats used in these studies (S. Munoz, unpublished). Furthermore, Swindle *et al* caution that our results showing cNOS expression and NO production in HMC-1 and LAD-2 may be related to the tumorigenic origin of these cell lines. However, we also identified mRNA for nNOS and eNOS in freshly isolated human skin MC (11) and a number of other studies have shown the expression of nNOS in *in vivo* derived MC populations [39,41]. The current study contributes to this debate by demonstrating PIN/LC8 in MC and an interaction between nNOS and PIN/LC8 in human MC lines, an interaction previously characterized in other cell types known to produce nNOS derived NO [30].

One NO regulated function that we previously identified was production of LT [11]. Interestingly, our preliminary results have identified inhibitory effects of IFN-γ, and upregulatory effects of IL-4, on LT production from HMC-1. However our hypothesis that blocking interactions between PIN/LC8 and nNOS would

decrease LT production was erroneous, and in fact the opposite was true. Furthermore, L-NAME had no effect on this anti-PIN/LC8 induced enhancement of Ca^{++} ionophore-induced LT production, indicating that the anti-PIN/LC8 enhancement effect was NO independent.

To understand this apparent paradox, it is important to consider potential activities of PIN/LC8 in MC. As a light chain of the cellular dynein complex, it binds several molecules besides nNOS. Immunoprecipitation, pepscan and yeast-two hybrid assays have identified over 20 proteins able to bind PIN/LC8 [27]. Furthermore, these binding partners have diverse roles within cells, including apoptosis, cellular metabolism, protein packaging and transport, and transcription [23,27,33]. Indeed, our preliminary results in HMC-1 have identified PIN/LC8 binding partners of similar diversity (unpublished results) indicating that the role of PIN/LC8 in MC is complex and not limited to modulating NO or its functions. Therefore, interfering with PIN/LC8 is likely to influence a number of proteins, particularly their location within the cell, as the primary function of cellular dynein is intracellular transport [23]. This includes nNOS, as it has been reported recently that PIN/LC8 is unable to inhibit NO production from nNOS in some settings, suggesting that one role of PIN/LC8 may involve intracellular transport of nNOS [42].

It is difficult to be specific about a mechanism to explain the anti-PIN/LC8 induced increase in LT release because of the diversity of potential protein partners and effects. The stimulus dependence of this (shown in Figure 2.5A, Column 7; non-A23187 stimulated population) further suggests that the proteins affected are

involved in the signaling responsible for LT production, or transport of LT to the cell periphery. Of note, LT are known to be exported to the cell surface after production via a specific energy-dependent step which requires the presence of multidrug resistance-associated protein 1 (MRP1) [6]. Interestingly, PIN/LC8 can bind myosin V [23] which is a member of the myosin superfamily responsible for transport of intracellular cargo to the cell surface [43]. It is possible that one step in this LT export mechanism involves an energy consuming transport system, such as myosin V, in which PIN/LC8 is a factor, and could explain why blocking PIN/LC8 affects LT export. Another explanation could be that PIN/LC8 is involved in regulating the transport of enzymes key to LT synthesis, such as 5-lipoxygenase or 5-lipoxyganse activating protein. Further studies must determine the full spectrum of PIN/LC8 binding partners before this type of idea is pursued.

In summary, we have identified expression of PIN/LC8 mRNA and protein in two human MC lines and demonstrated that this expression can be influenced through exposure to T_h1 and T_h2-type cytokines. Additionally, we have shown that PIN and nNOS are physically associated. Interestingly, anti-PIN/LC8 antibodies potentiate LT release in a NO-independent manner, although how this occurs is unknown. To understand the undoubtedly diverse roles of PIN/LC8 in MC, future studies must focus on identification and characterization of PIN/LC8-protein interactions.

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Chapter 3

Identification of Putative PIN/LC8 Binding Partners in HMC-1

Introduction

Cellular dynein is a large multicomponent complex responsible for intracellular transport of various cargo along the microtubule (MT) network within cells [1]. MT are polar, and generally are arranged with their 'plus' ends radiating away from the nucleus [1]. Dyneins move unidirectionally along MT towards the 'minus' ends, and thus generally transport their cargo towards the nucleus [2] (Fig. 1.1).

The dynein complex is made up of a number of different proteins. This includes two heavy chains (HC) responsible for the generation of the motor force through hydrolysis of ATP, two intermediate chains (IC) which are important in regulating dynein function, and two light intermediate chains (LIC) [3]. Additionally, there is a variable number of light chains (LC) associated with the dynein motor complex, and these LC are the primary binders of dynein's cargo [2].

The LC with the most identified binding partners is LC8. Interestingly, LC8 was also identified as an inhibitor of neuronal nitric oxide synthase (nNOS), and was termed protein inhibitor of nitric oxide synthase (PIN) [4]. Duplication of this inhibitory effect on nNOS has provided variable results [5,6] however, and it is now suggested that PIN/LC8 functions as a transporter of nNOS, not an inhibitor [6].

PIN/LC8 also binds over 20 additional proteins and is believed to be a major player in intracellular transport. These cargo can include enzymes, transcription factors, signaling molecules, and apoptosis related proteins [7]. PIN/LC8 is also reported to bind a number of viral proteins and is required for their transport to the

nucleus [8]. Moreover, PIN/LC8 has been shown to associate with another motor protein, myosin V, responsible for transport along actin filaments in the cytoskeleton [9]. Proper functioning of PIN/LC8 is important to numerous cellular processes.

Mast cells (MC) are tissue-resident cells which arise from immature precursors in the bone marrow and undergo final maturation at their tissue location [10]. They are capable of releasing stored mediators, as well as newly synthesizing bioactive agents, upon activation. Due to their unique armamentarium of mediators and their location at tactical locations in the body, MC are implicated in numerous disease states including inflammatory bowel disease [11], fibrosis [12], autoimmune disease [13], and allergic diseases such as asthma [14].

Our recent work has described the expression of PIN/LC8 in MC, and showed its ability to bind to nNOS. The purpose of this study is to begin to describe what other proteins associate with PIN/LC8 in MC, and suggest potential roles for this interaction in MC function.

Materials and Methods

Cell Line:

The immature human MC line, HMC-1 (a gift from J.H. Butterfield, Rochester, MN) was cultured in Iscove's medium (Life Technologies, Grand Island, NY) with 10% fetal bovine serum (FBS), 2 mM glutamine, 40 U/ml penicillin/streptomycin and 1 mM thioglycerol. Cells were maintained at 37°C in a humidified incubator at 5% CO_2.

GST-PIN protein:

The generation and purification of recombinant GST-PIN/LC8 and GST control were performed as previously described in chapter II. The pull-down experiments were also done as in chapter II, but after elution with glutathione elution buffer, eluant was mixed with 3x SDS Sample Buffer (New England Biolabs, Beverly, MA) and separated on a 10-20% gradient pre-cast SDS-PAGE gel (BioRad, Hercules, CA). The gel was then silver stained as described below.

Silver-Staining and Band Excision:

The 10-20% gradient pre-cast gel was subject to silver stain visualization using a Plus One silver stain kit (Amersham, Buckinghamshire, UK) according to manufacturer's protocol. All staining solutions were placed in a stainless steel container on a rocker. The gel was exposed to the fixing solution (100 mL ethanol, 25 mL acetic acid, and 125 mL water [H_2O]) for 30 min. The fixing solution was removed, and the gel was immediately exposed to the sensitizing solution (75 mL ethanol, 0.012 M sodium thiosulphate, 0.83 M sodium acetate, and 175 mL H_2O) for

30 min. The sensitizing solution was removed, and the gel washed three times for 5 min in H_2O. The gel was then exposed to the silver solution (0.015 M silver nitrate and 0.005 M formaldehyde in 250mL distilled water) for 20 min, then washed for 1 min in distilled water. The developing solution (0.24 M sodium carbonate and 2.5 mM formaldehyde in 250 mL distilled water) was then added for approximately 5 min, until the bands were easily visualized. The stopping solution (0.04 M EDTA-Na_2-2 H_2O in 250 mL distilled water) was then added for 10 min to end the developing reaction. The gel was then washed for 10 min in distilled water before five bands of significant abundance were excised by hand using a razor blade, and placed in H_2O in a 1.5 mL eppendorf tube for storage before tryptic digestion.

In-Gel Tryptic Digestion and Liquid Chromatography (LC)/Mass Spectometry (MS):

All in-gel tryptic digestion, liquid chromatography, and mass spectrometry were performed at the Institute for Biomolecular Design, University of Alberta, Edmonton, Alberta, Canada. In-gel tryptic digestion was performed on the excised bands using a Mass Prep Station (Waters, Milford, MA). The gel pieces were de-stained, reduced (DTT), alkylated (iodoacetamide), digested with trypsin (Promega Sequencing Grade Modified) and the resulting peptides extracted from the gel and analyzed via LC/MS. LC/MS was performed on a CapLC HPLC (Waters) coupled with a Quadropole-Time of Flight (Q-ToF)-2 mass spectrometer (Waters). Tryptic peptides were separated at a flow rate of 200-400 nl/min using a linear water/acetonitrile gradient (0.2% formic acid) on a Picofrit reversed-phase capillary column, (5 micron BioBasic C18, 300 Angstrom pore size, 75 micron ID x 10 cm,

15 micron tip) (New Objectives, Cambridge, MA), with an in-line PepMap column (C18, 300 micron ID x 5 mm), (LC Packings, Sunnyvale, CA) used as a loading/desalting column. The column was hooked up directly to the Q-ToF-2 mass spectrometer.

Peptide Identification:

Protein identification from the generated MS data was done searching the National Center for Biotechnology Information (NCBI) non-redundant database using Mascot Daemon (Matrix Science, London, UK). Search parameters included carbamidomethylation of cysteine, possible oxidation of methionine and one missed cleavage per peptide. The data being analyzed are the mass X of the intact peptide and the associated fragmentation spectrum. The theoretical fragmentation spectra from all peptides in the database having the same mass X (+/- some tolerance) are compared with the observed fragmentation pattern, and a match with an associated confidence correlation is given. A confidence correlation value greater than 47 indicates identity or extensive homology.

Results

After performing the GST-PIN/LC8 pulldown, SDS-PAGE, and silver stain, five separate bands were identified, cut out of the gel, and named based on their approximate molecular weight. The five names were "30 kDa", "39 kDa", "50 kDa", "55 kDa", and "74 kDa" (Fig. 3.1). After MS analysis of these bands, their identity was determined as described in "Materials and Methods" and is shown in Table 3.I. All identities were established by having confidence correlation values greater than 47.

The "30 kDa" band was identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), with a confidence correlation value of 135. The actual molecular weight of GAPDH is 36 kDa, which matches with the location of the band excised from the gel.

The "39 kDa" band was identified as β-actin with a confidence correlation value of 310. The actual molecular weight of this protein is 42 kDa and therefore matches with its location on the gel.

The "50 kDa" band was positively identified, with confidence correlations from 560-50, as both albumin and keratin, both whose actual molecular weights range from 40 to 60 kDa. It is difficult to specify which protein this band actually represents, and is likely contamination either from human or cell culture media.

The "52 kDa" band was positively identified as α-1-antitrypsin (AAT), with a confidence correlation of 193. The actual molecular weight of this protein is 46 kDa, and matches the location of the excised band.

The "75 kDa" band was positively identified as heat shock protein (HSP)-90α, with a confidence correlation of 208. The actual molecular weight of this protein is 85 kDa and is thus at a molecular weight corresponding to the location of the excised band.

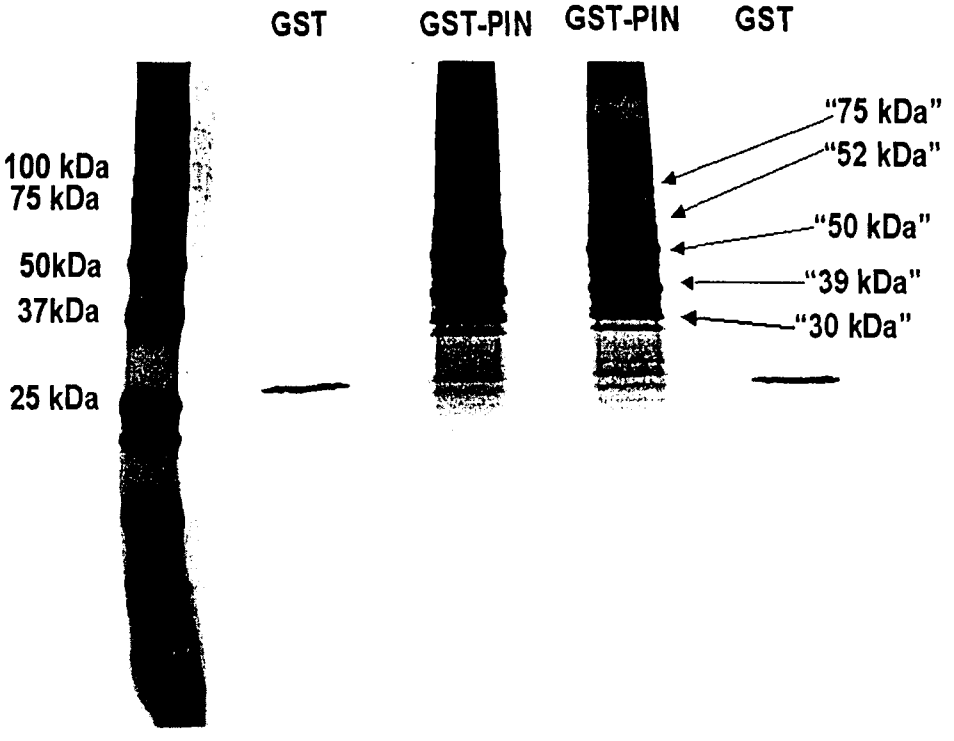

Fig. 3.1: Total number of proteins that bind PIN/LC8 in HMC-1. Silver stain analysis of a 10-20% precast gel following SDS-PAGE of 30 μL of GST-PIN/LC8 and GST eluants following pulldown analysis of HMC-1. Indicated bands were excised and stored for LC/MS analysis. Samples were run in duplicate to ensure sufficient protein abundance for identification. Representative of two independent experiments. Mass spectrometry was run in duplicate from the second experiment.

TABLE 3.1: Results of protein identification acquired following GST-PIN/LC8 pulldown and LC/MS of HMC-1 lysate.

BAND	PROTEIN	CONFIDENCE CORRELATION	PRESENCE OF PIN/LC8 BINDING SEQUENCE	PREVIOUSLY IDENTIFIED AS A PARTNER FOR PIN/LC8?
"30 kDa"	GAPDH	135	YES	YES
"39 kDa"	β-Actin	310	NO	YES
"52 kDa	α-1-antitrypsin	193	NO	NO
"75 kDa"	HSP-90α	208	YES	NO

Discussion

For PIN/LC8 to bind a cargo protein, it is expected that the cargo must contain a putative PIN/LC8 binding site. There are two known sequences that can make up these binding sites. One such site is denoted by the consensus sequence (K/R)XTQT, where X can be any aa [15]. Examples of proteins containing this sequence include the Bcl-2 family protein, Bim, the transcription factor Kid-1, and a number of different viral proteins [16]. The other consensus sequence for PIN/LC8 binding reads G(I/V)QVD [17], and is found in nNOS and guanylate kinase domain-associated protein (GKAP) [16].

Interestingly, two of the proteins we identified as binding PIN/LC8 in HMC-1 contain sequences that are similar to these consensus sequences, which suggests direct binding to PIN/LC8. HSP-90α contains the aa sequence KEDQT, and greatly resembles the (K/R)XTQT motif, the only difference being the threonine (T) residue is replaced by an aspartate (D) residue. This may not be of much concern with regard to PIN/LC8 binding, as it has been established that in most cases the glutamine (Q) residue is critical, and is followed closely by beta-branched aa residue, such as T [7]. This sequence found in HSP-90α satisfies these requirements and suggests direct binding to PIN/LC8.

GAPDH also contains a sequence which resembles the PIN/LC8 binding sequence, TATQKT. This sequence differs slightly from the (K/R)XTQT motif, but it maintains two key elements: 1) the conserved Q residue, and 2) the beta-branched residue following it, in this case a T two residues after. This suggests that GAPDH possesses a binding sequence similar to a known PIN/LC8 binding motif

It is likely the "50 kDa" band that was strongly identified as both albumin and keratin is a result of contamination from either human skin or the FBS which is a component of the HMC-1 cell culture medium. Sequence analysis of both proteins resulted in no regions containing homology to either consensus pattern for PIN/LC8 binding, suggesting that these proteins were present as contaminants because of their abundance in the experimental prep and incomplete washing, and not from direct PIN/LC8 interactions.

β-actin is a component of actin filaments which make up the MC cytoskeleton. AAT is a protease inhibitor that has been identified in MC [18]. Both of these proteins were identified in the GST-PIN/LC8 pulldown with high confidence correlation values (310 and 193, respectively), but neither protein contains a version of the putative PIN/LC8 binding motif. Other studies looking at PIN/LC8 protein interactions have seen similar results. For example, IκB, which regulates the transcription factor NFκB, can directly associate with PIN/LC8, but does not contain a canonical LC8-binding sequence [19]. It has been suggested that proteins capable of interacting with other proteins, such as IκB, may contain other regions dedicated to this function, and might facilitate PIN/LC8 interactions this way [7]. These may include PDZ or SH3 domains. Alternatively, proteins can be "second-hand" bound to PIN/LC8 through associating with various linker proteins, which directly bind PIN/LC8 [7]. In the case of β-actin and AAT, both of these potential mechanisms may explain their PIN/LC8 interactions.

The interaction between GAPDH and PIN/LC8 may have physiological relevance. GAPDH is a key enzyme in the glycolytic pathway, which is a major

ATP producing metabolic process within cells. Specifically, it catalyzes conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. Interestingly, other enzymes involved in this pathway in addition to GAPDH, such as aldolase, pyruvate kinase, and lactate dehydrogenase, have also been found to cluster around microtubules [20]. It makes sense that enzymes involved in the same pathway would be in close vicinity to each other to facilitate an efficient operation. Indeed, GAPDH, lactate dehydrogenase and another glycolytic enzyme, phosphofructokinase-1, have been shown to bind to PIN/LC8 in rat brain [7]. This suggests a mechanism involving PIN/LC8 and dynein coordination of these enzymes to initiate or aid in this process. This may be a common theme amongst a number of cellular pathways, as two enzymes from the citric acid cycle, citrate synthase and malate dehydrogenase, were also identified as PIN/LC8 cargo [7].

Similarly, it is not difficult to rationalize an interaction between PIN/LC8 and HSP-90. HSP-90 is one of the most abundant and multifunctional cellular proteins, primarily responsible for correct folding and maturation of countless proteins [21]. These include various pluripotent signaling proteins involved in transducing the signals from diverse receptors and stimuli. HSP-90 is also important in the preparation of many transcription factors, including p53, Stat3, and a number of steroid receptors [21]. It follows that intracellular location of all these proteins is crucial to their function and that their movement within cells could be dependent on molecular motors such as dynein, or myosin, depending on their purpose. Such a process could involve PIN/LC8 as the linker to these HSP-90/protein complexes and direct their intracellular migration.

Interestingly, HSP-90 has also been shown to be involved in the activation of both eNOS [22] and nNOS [23]. The evidence that PIN/LC8 binds nNOS is quite convincing [4,7,24] (and data in this thesis), and this provides a potential mechanism by which HSP-90 and nNOS may be brought together. Furthermore, in MC eNOS is located mainly in the nuclear region of the cell [25]. HSP-90 may therefore depend on PIN/LC8 mediated transport for its translocation to the nucleus to foster its interactions with eNOS.

AAT association with PIN/LC8 may also have bearing on its function. AAT is a protein known to inhibit the activity of a number of proteases, including neutrophil elastase, cathepsin G, and proteinase 3 [26]. Additionally, MC chymase can be inactivated by AAT [27]. AAT is heavily implicated in the development of emphysema, as patients lacking the enzyme are susceptible to lung tissue degradation by neutrophil elastase [26]. Proteases are a major component of MC and are key pathological mediators in MC associated conditions. Furthermore, these proteases appear to have a role in the ability of MC to exocytose, as histamine release from human MC was significantly inhibited after exposure to a number or protease inhibitors, including AAT [28]. This suggests that AAT found in MC may be directed to locations of protease storage to maintain proper function of these cells. It appears that a limited number of cell types are able to synthesize AAT [26], and MC are not included in this group. If this is the case, PIN/LC8 could play an important role of moving endocytosed AAT to protease storage locations within MC.

The association of β-actin with PIN/LC8 may not have functional relevance. Instead, it is likely that some β-actin was bound to myosin within the MC lysate and the GST-PIN/LC8 pulled down myosin with the contaminating β-actin, which were then subsequently dissociated in the SDS-PAGE procedure. Further studies must be performed to confirm this hypothesis, as PIN/LC8 has yet to be shown to bind myosin in MC.

The evidence presented in this chapter confirms that PIN/LC8 has a large number of binding partners, all involved in critical cellular processes. Further work is required to unravel the undoubtedly complex role PIN/LC8 has in regulating MC function.

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Chapter 4

General Discussion and Future Directions

I. Discussion

A. Summary of data

The light chain component of the dynein complex, PIN/LC8, is a key molecule in the recruitment and linking of numerous proteins to the molecular motor for efficient retrograde transport within cells. One such protein, nNOS, is potentially a key regulatory protein to MC functions, as NO is able to regulate a number of these functions. The overall objectives of this thesis were to define PIN/LC8 expression, regulation, and function in human MC. PIN/LC8 mRNA and protein were found to be expressed in two human MC lines, HMC-1 and LAD-2. Furthermore, PIN/LC8 expression was differentially regulated by T_h1 (IFN-γ) and T_h2 (IL-4) cytokines. PIN/LC8 mRNA was similarly regulated in both cell types by these cytokines, but the protein levels in these two cells were influenced in different ways by the same cytokines. Additionally, potential protein binding partners of PIN/LC8 in MC were identified. These included GAPDH, AAT, HSP-90, and nNOS. Finally, intracellular delivery of antibodies to PIN/LC8 potentiated LT production and release in a stimulus dependent and NO-independent fashion.

B. Regulation of PIN/LC8 expression in mast cells

The observation that PIN/LC8 expression could be influenced by cytokines was first shown in our lab by gene array analysis of HMC-1 following their exposure to IFN-γ. This experiment showed a decrease in PIN/LC8 mRNA expression. Interestingly, this treatment also resulted in the upregulation of eNOS.

Subsequent studies have shown the extensive effects of IFN-γ on NOS expression and NO production in MC [1-3].

Initially, the goal of my study was to examine PIN/LC8 expression in the context of NO production and regulation in MC. However, after careful review of the literature, two key things became apparent: 1) that PIN/LC8 was only able to bind to the neuronal NOS isoform, and was unable to disrupt its production of NO [4,5]; and 2) that PIN/LC8 was not limited in its binding to nNOS, but was capable of binding to numerous different proteins [6]. These observations suggest that PIN/LC8 is a key molecule in the general functioning and survival of cells, and that attempting to limit a definition of PIN/LC8 activity to one cellular function is inappropriate.

Treatment of MC with cytokines that act in a reciprocal fashion to IFN-γ with respect to NO production, namely T_h2 cytokines IL-4 and IL-10, followed the hypothesis that such cytokines would have the opposite effect on PIN/LC8 expression and could be linked to decreased NO production. Though it has become apparent that PIN/LC8 has a limited role in NO production from nNOS, the regulation of expression of PIN/LC8 is key to the proper functioning of these cells. Given that PIN/LC8 can bind to proteins like the proapoptotic Bcl-2 family protein, BIM [7], and the key inhibitory element of the transcription factor NFκB, IκB [8], what role do cytokines that modulate PIN/LC8 expression play in protein synthesis and regulated cell death of MC?

Table 4.1 summarizes the change in PIN/LC8 mRNA and protein expression in the two MC lines, HMC-1 and LAD-2, after treatment with cytokines. The

mRNA expression changes similarly in the two cell types, with IFN-γ decreasing and IL-4, and IL-10 to a lesser extent, increasing PIN/LC8 message. As suggested in chapter II, these differences may be attributed to the differences in relative maturity of these two cell lines, as LAD-2 display a more mature and differentiated

Table 4.1: Summary of cytokine regulation of PIN/LC8 mRNA and protein expression in mast cells

Cell Type	Readout	IFN-γ	IL-4	IL-10
HMC-1	mRNA	↓	↑ *	↑
	Protein	↓ *	↓ *	↑
LAD-2	mRNA	↓	↑	↑
	Protein	—	↑ *	↑

* indicates statistically significant change in expression.

phenotype than HMC-1 and have a much slower turnover rate (2-3 weeks compared to 80 h in HMC-1) [9].

The change in protein levels between the two MC populations differed significantly, however (Table 4.1) IFN-γ had little effect on LAD-2, but decreased PIN/LC8 levels in HMC-1 ($P<0.001$; Fig. 2.3 A). IL-10 had little effect on both cells, though slightly increased PIN/LC8 in LAD-2. IL-4 induced regulation of the protein in complete contrast between the two cells, significantly decreasing

PIN/LC8 in HMC-1 ($P<0.001$; Fig. 2.3 A) while increasing it in LAD-2 ($P<0.001$; Fig. 2.3 B). These differences may be due to differences in phase of maturation as it has been shown that MC respond differently to IL-4, including in protein expression, depending on their maturity [10]. Furthermore, in rat muscle cells PIN/LC8 is more abundant in embryonic muscle fibers than adult muscle [11], and partial loss-of-function mutations of PIN/LC8 in *Drosophila* are associated with defective wing development [12]. This type of data suggests PIN/LC8 plays a role in the development of these cells, and therefore this protein has potentially different functions at different stages of cell development. The idea can be extended to MC, where PIN/LC8 may have different functions in more immature HMC-1 than in the more mature LAD-2, which is highlighted by its opposite regulation by IL-4, and differing regulation by both IL-10 and IFN-γ.

C. PIN/LC8 binding partners in mast cells

The spectrum of PIN/LC8 binding partners is constantly expanding. To date, over 20 different proteins have been identified, with novel allies continuously being described. This thesis has begun to address the portfolio of PIN/LC8 associated proteins in the MC. Considering the broad role MC play in human physiology and disease, it is essential to know what this molecule is doing in MC functions. Two experimental methods were used to demonstrate PIN/LC8 binding; western blot analysis (chapter II), and mass spectrometry (chapter III). However, both techniques used recombinant glutathione-S-tranferase (GST)-tagged PIN/LC8 molecule to perform pulldown assays from MC lysates. This technique allows for the potential

identification of all proteins in the cell able to bind PIN/LC8, but does not identify the location or regulation of these interactions as cells are lysed prior to exposure to GST-PIN/LC8. Further studies employing confocal microscopy and transfection of MC with fluorescently labeled PIN/LC8 would allow for determination of it subcellular location and relationship to its cargo.

However, my initial studies have identified new candidates for study and have generated new hypotheses regarding PIN/LC8 in MC, such as PIN/LC8 is involved in protein synthesis and sorting due to its interaction with HSP-90. It will be important to confirm my results that showed nNOS interaction with PIN/LC8 in MC. As human MC are reported to only express the eNOS and nNOS isoforms [3], determining why and when nNOS is bound to PIN/LC8 will help provide hypotheses that can be tested to explain the stabilizing effects of NO on MC.

Extending this idea, my observations that identified for the first time in MC that HSP-90, GAPDH, and AAT are PIN/LC8 binding proteins provide a framework for several studies. Considering the critical "housekeeping" roles both HSP-90 and GAPDH, determining where and when they are bound to PIN/LC8 will provide a great deal of insight to the inner workings of these unique cells.

D. <u>Effect of blocking PIN/LC8 on leukotriene production</u>

Our previous work showed that production of LT from MC could be attenuated by treatment with exogenous NO donors [3]. Furthermore, we identified a potential role for eNOS in the regulation of LT production, as the LT generating enzyme, 5-LO, was found co-localized with eNOS associated with the nucleus of

activated MC [3]. Results from this study found a largely cytoplasmic staining for nNOS, which appeared to be unchanged following MC activation with A23187.

However, our finding that PIN/LC8 binds nNOS prompted us to investigate what effect interfering with this interaction would have on LT production. As endogenous NO produced by eNOS was never conclusively shown to be the LT regulating unit, it was hypothesized that nNOS not bound to PIN/LC8 would make NO, which would mimic the inhibitory effects of exogenous NO on LT production.

Unexpectedly, the opposite effect was found. Disruption of the PIN/LC8-nNOS complex by anti-PIN, confirmed by pre-incubation of GST-PIN/LC8 with the anti-PIN/LC8 prior to pulldown analysis (Fig. 2.4 A), caused a significant increase in LT production, rather than the predicted decrease. The stimulus (A23187) dependence of this response, shown in Fig. 2.5 A; column 7, suggested that it could involve a NO dependent phenomenon, as eNOS and nNOS require increased intracellular [Ca^{2+}] to function.

To test this theory, anti-PIN/LC8 antibodies were introduced, but prior to stimulation with A23187, cells were treated with the NOS inhibitor L-NAME. If the observed increase in LT produced was NO-dependent, pre-treatment with this inhibitor should eliminate the effect. However, Fig. 2.6 highlights the results showing that production of LT was unaffected by L-NAME, indicating the anti-PIN/LC8 increase in LT was NO-independent.

As mentioned above, there are numerous binding partners of PIN/LC8 beyond nNOS, and these include proteins of diverse function. It is apparent that blocking PIN/LC8 binding has a significant effect on LT production, but how this

effect is propagated has not been established. The mechanisms of LT transport from the perinuclear site of its synthesis [13,14] to its extracellular release are largely unknown, apart from the data that it requires MRP-1 [13]. As discussed in chapter II, the export of LT from the nuclear envelope is energy dependent, suggesting that a dedicated transport machinery, such as that involving myosin and PIN/LC8 is required for LT transport and secretion.

Our data suggests that PIN/LC8 binding partners have a role in LT release from MC, though in an NO-independent fashion. Identifying other PIN/LC8 cargo in MC will help determine the mechanism of LT production and secretion that involves PIN/LC8, and will also help uncover the broader roles of PIN/LC8 in MC biology. These issues are addressed in chapter III, as well as later in this chapter.

II. Future Directions

A. Identification of binding partners of PIN/LC8 in mast cells

The ability of PIN/LC8 to interact with various proteins and facilitate their transport within cells appears to be a crucial event in cell biology. The apparently unrelated nature, apart from containing a conserved PIN/LC8 binding motif, yet critical function of these proteins makes their identification an important priority. The portfolio of PIN/LC8 binding partners may differ among cell types, and their identification may provide new insight to cellular processes. Indeed, in MC the number of PIN/LC8 interacting proteins is large (Fig 3.1).

Experimentally, an effective way to identify these proteins is by using a recombinant PIN/LC8 protein that has been tagged with a moiety that facilitates purification, such as a histidine tag, or in our case, a glutathione-S-transferase (GST) tag. Pulldown experiments from cell lysates can be performed, followed by purification of the recombinant PIN/LC8, separation of all binding proteins by SDS-PAGE, and identification by mass spectrometry. Although this is particularly effective for the identification of novel binding proteins, the technique can uncover a large number of proteins to identify (Fig. 3.1). To minimize this problem, one could perform subcellular fractionation prior to the PIN/LC8 pulldown experiment. This could potentially reduce the number of PIN/LC8 binding proteins, and provide location-specific data regarding what binds PIN/LC8. Alternatively, the pulldown experiments could be followed by western blot analysis to locate a specific protein, as was done for nNOS in chapter II. This is effective only if there is some indication

that the protein of interest is capable of this interaction, such as the presence of a PIN/LC8 binding motif [15,16].

B. Determination of intracellular location of PIN/LC8 interactions

Identifying the full spectrum of proteins that PIN/LC8 interacts with is one part of investigating how PIN/LC8 operates *in vivo*. Given that the fundamental function of the dynein complex is intracellular transport [17], determining where, and under what conditions, these interactions occur is vital to understanding the roles of PIN/LC8. For instance, the proapoptotic Bcl-2 family protein, Bim, has been established to be a PIN/LC8 partner which under healthy cellular conditions, in human embryonic kidney (HEK) 293 cells, is sequestered to dynein and myosin complexes by PIN/LC8 [7]. However, when the cells are exposed to apoptotic stimuli, such as ultraviolet light, the Bim-PIN/LC8 complex is found at the mitochondria and nucleus where Bim can exert its apoptotic effects. This highlights the importance of subcellular PIN/LC8-protein interactions, and most certainly applies to numerous other PIN/LC8 cargo.

Although such studies can be challenging, one potential procedure is the stable transfection of the cell of interest with a plasmid encoding a functional PIN/LC8 tagged with a fluorescent tag, such as green fluorescent protein (GFP). This would allow for the visualization of PIN/LC8 intracellular location under different experimental conditions using live cell confocal microscopy. Additionally, these cells could be fixed and stained with antibodies against the cargo protein of interest, looking for co-localization of GFP-PIN/LC8 and cargo under experimental

treatments. However, this technique may be limited by the small size of PIN/LC8 (~10 kDa), and the larger size of fluorescent tags like GFP whose molecular weight is approximately 3 times that of PIN/LC8 (~25-27 kDa).

Interestingly, a recent study identified a novel PIN/LC8 cargo to be p53 binding protein 1, which facilitates the dynein-dependent migration of tumor suppressing p53 to the nucleus under conditions of DNA damage [18]. The investigators looked at the ability of Tat-tagged synthetic peptides corresponding to PIN/LC8 binding motifs to impede GFP-p53 migration to the nucleus. Tat is a membrane penetrating protein of the HIV virus, and is commonly used to introduce peptides into living cells [19]. This technique allows for the examination of PIN/LC8 intracellular interactions, and is especially effective if the cargo of interest is fluorescently labeled, or if its function can be easily monitored, for instance by measuring induction of Bim-mediated apoptosis using the annexin V assay. Alternative to Tat-tagged peptides, introduction of antibodies specific to PIN/LC8 using a bioporter tool, such as Chariot in chapter II, could be used for these studies.

C. IFN-γ and IL-4 effects on mast cells. Role for PIN/LC8?

The results in chapter II indicate that both IFN-γ and IL-4 can regulate expression of PIN/LC8 mRNA and protein in MC. It follows that these cytokines could potentially regulate the many cellular processes that involve PIN/LC8. For instance, chapter II mentions preliminary experiments showing that these cytokines can regulate LT production, with IL-4 enhancing and IFN-γ inhibiting their production. These experiments were an extension to those performed by Gilchrist *et*

al [3] who showed that NOS inhibitors increased and NO donors inhibited LT production. Since IL-4 and IFN-γ can influence NO production in MC [2,20], and we established in chapter II that they regulate the expression of PIN/LC8, we hypothesized that the amount of PIN/LC8 present would influence NO production through the PIN/LC8 interaction with nNOS, and therefore adjust LT production accordingly. However, our αPIN/LC8 blocking experiments resulted in a dramatic, NO-independent, potentiation of LT production from MC (chapter II; Fig. 2.5A). Knowing the extensive role PIN/LC8 plays in cell biology, its regulation by these two hallmark T_h1 and T_h2 cytokines suggests that PIN/LC8 plays a central role in their effects on MC responses.

D. <u>A potential novel role of nNOS in regulating mast cell function. Is PIN/LC8 involved?</u>

As outlined in chapter I, NO is recognized as a signaling molecule, and through its ability to nitrosylate and nitrate proteins it is involved in modulation of signaling cascades in the manner reminiscent of phosphorylation [21,22]. Furthermore, each NOS isoform produces NO with differing functions. Recent work investigating myocardial contractility of mice identifed nNOS derived NO as a key regulator of the intracellular release of Ca^{2+} from storage in the sarcoplasmic reticulum (SR), likely through the nitrosylation of the ryanodine receptor (RyR) [23]. Interestingly, the superoxide (O_2^-) producing enzyme xanthine oxidoreductase (XOR) can occur in close physical proximity to nNOS in the SR [24]. O_2^- can be an important inhibitory signaling molecule in the Ca^{2+} driven muscle responses in

myocardium [25]. NO and O_2^- are capable of combining and forming peroxynitrite ($ONOO^-$) [26], which does not appear to play a role in regulating Ca^{2+} mediated events in these cells. An innovative model has been described where XOR and nNOS are closely associated in the SR and regulate one another, thus influencing the contractility of the muscle [24]. Indeed, the two enzymes co-immunoprecipitate with each other. It is important to note that only nNOS is involved in this regulation, as XOR does not appear to associate with eNOS in these cells [24]. The model proposed states that when nNOS and XOR are in close proximity, the relative activity of the two enzymes is able to regulate Ca^{2+} release from the SR, and thus muscle contraction. Therefore, when XOR produces high [O_2^-], it is able to decrease muscle contraction by reacting with nNO to produce $ONOO^-$, as well as carrying out its inhibitory signaling. Likewise, when nNOS makes high [nNO], the amount of free O_2^- is reduced and the RyR is nitrosylated, allowing for increased Ca^{2+} release. Furthermore, XOR contains a number of redox centers which could be susceptible to nitrosylation, further decreasing O_2^- production [24] (Fig 4.1). Indeed, O_2^- production was greatly increased in nNOS knockout mice when compared to wild type or eNOS knockout mice [24].

It is attractive to suggest this model could be applied to MC. As described in chapter I, when MC are classically activated through FcεRI, the signaling cascade leads to the release of Ca^{2+} from intracellular stores in the endoplasmic reticulum (ER) via the IP3 receptor, which is a critical step in granule exocytosis [27]. Additionally, recent *in vivo* studies using NOS knockout mice have demonstrated a key role for nNOS,

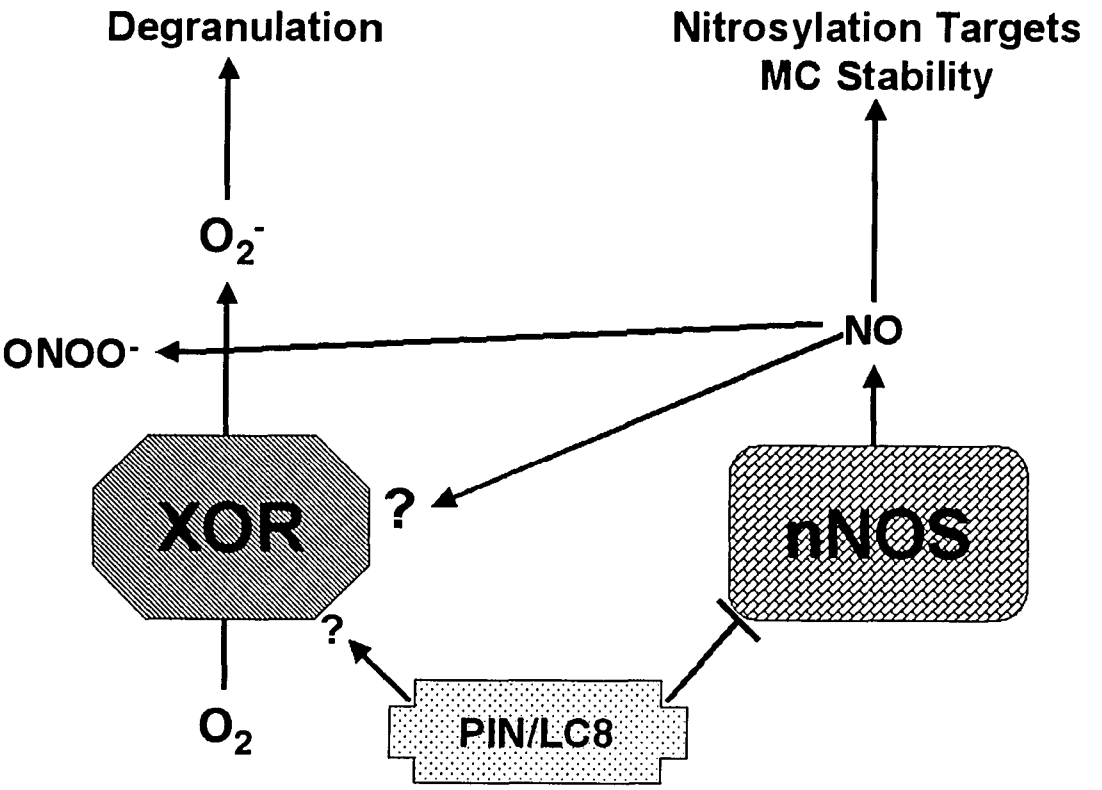

Fig. 4.1: Potential interaction between nNOS and XOR which results in the seesaw regulation of production of the activating superoxide (O^{2-}) and the inhibitory nitric oxide (NO).

but not eNOS or iNOS, in the development of airway hyperresponsiveness [28]. Determining what relationship XOR and nNOS have in MC, and if this model could possibly apply in MC is of particular interest. Furthermore, given that PIN/LC8 is capable of binding to nNOS, it may regulate this potential interaction by facilitating nNOS movement within the cell to associate with XOR. To examine this experimentally, western blot analysis for XOR, as well as co-immunoprecipitation and GST-PIN/LC8 pulldown experiments could be performed to search for a physical association between these molecules. Immunostaining, followed by confocal microscopy could also be used to confirm co-localization. Further studies could involve monitoring the production of NO and O_2^- from MC when stimulated to degranulate to investigate what the predominant reactive species being produced is. Indeed O_2^- production has been correlated to MC degranulation [29], while NO production has been correlated with MC stability [2]. Furthermore, it has been shown that NO production by activated MC is heterogeneous, with a percentage of MC not producing NO and degranulating in response to stimuli, and a percentage that strongly make NO and are non-responsive [2]. It is exciting to consider that a similar mechanism of XOR and nNOS regulation occurs in MC, and that individual cell responses to the same stimuli can be attenuated in this fashion.

It should be noted that for this model to apply in MC, the NO would not likely be involved in increasing Ca^{2+} release from the ER, as this would cause degranulation. Instead, NO would likely act to stabilize the MC. This could include a nitrosylation event of a critical substrate such as IP3R that would hinder effective IP3 binding, or could involve NO-mediated inhibition of other critical signaling

events. In fact, NO has been shown to inhibit degranulation, as well as the activation of critical signaling molecules involved in cytokine production in MC [30]. Furthermore, nNOS derived NO has specifically been shown to block Ca^{2+} induced signal transduction in HEK 293 cells [31]. It should be noted that nNOS itself can produce O_2^- in the absence of certain co-factors [32], so the level of regulation may be more complex than a simple "seesaw" mechanism.

Nonetheless, the evidence suggests that both O_2^- and NO are capable of modifying MC activity, and the model of close proximity production of both reactive species balancing one another which then in turn has an impact on MC reactivity is novel and worth pursuit. To be sure, PIN/LC8 could have a role in guiding nNOS within the cell, so that its proximity to XOR would be relevant.

E. A role for PIN/LC8 in mast cell granule exocytosis?

This thesis has addressed what effect blocking PIN/LC8 protein interactions has on the production and release of LT from MC. This, however, is only one branch of the MC armamentarium. Another crucial event following MC activation in the exocytosis of secretory granules (degranulation) which contain various mediators, including histamine and proteases [33]. This was not addressed here for two major reasons: 1) HMC-1, the predominant cell line used, does not appear to form complete granules or undergo classical degranulation events. This is likely due to their rapid growth and immature status; 2) LAD-2, though they do appear capable of granule exocytosis, do not express nNOS [3], and determining PIN/LC8 interactions with nNOS was a major objective in this study.

A potential strategy to address this dilemma is the development of cord-blood derived human MC. This MC population is able to be activated through FcεRI, and can undergo degranulation, as well as produce lipid mediators and cytokines [34]. These cells provide a valuable model for the study of MC biology.

Recent work in the rat MC line, RBL-2H3, has shown the requirement for an intact microtubule network for exocytosis to occur [35]. Additionally, in epithelial cell lines, PIN/LC8 has been established as an important component in the control of vesicle formation during the endocytic process of macropinocytosis, which occurs in the actin framework of cells [36]. These studies help establish both the importance of microtubules to exocytotic events in MC, and implicate a role for PIN/LC8 in associating with vesicles in cells. Since PIN/LC8 is coupled to the major molecular motor complexes within these two cytoskeletal networks, dynein and myosin, it is possible that blocking PIN/LC8, using antibody delivery as in chapter II or synthetically generated peptides as mentioned above, would influence the degranulation potential of stimulated MC. The use of cord-blood MC would also allow for studies of the role PIN/LC8 plays in release of stored mediators, or newly synthesized lipid mediators or cytokines and chemokines.

F. Conclusions

Preliminary studies identified PIN/LC8 as an IFN-γ regulated gene in HMC-1. Subsequent work in this thesis has confirmed this, and shown that IL-4 and IL-10 regulate its expression. Interestingly, these cytokines are able to regulate NO production in MC. Furthermore, data in this thesis demonstrates the ability of PIN/LC8 to bind nNOS. However, PIN/LC8 is involved in numerous cell processes, as can be shown by its ability to bind a variety of cargo ([6], and chapter III). Interfering with PIN/LC8 binding is likely to affect a number of MC processes, highlighted in this study by the NO-independent potentiation of LT release.

Much work is still to be done investigating the complete role of PIN/LC8 in MC, including characterization of cargo, the location and conditions when these interactions occur, and what each of these interactions means to MC function. Knowing that MC are involved in many disease states, as well as in beneficial physiological processes, a complete understanding of the activities of PIN/LC8 is critical.

Additionally, the literature surrounding NO mediated regulation of MC functions is significant, making PIN/LC8-nNOS interactions potentially relevant. A model is presented in this chapter outlining a potential seesaw mechanism of self regulation between nNOS and superoxide producing XOR, which has significant implications on MC biology, and in which PIN/LC8 may have a role.

Overall, PIN/LC8 is a ubiquitous and conserved molecule, whose functions are vital to the healthy development, survival, and operation of many cell types. The involvement of MC in such diverse biological events such as allergic disease, host

defence, and homeostasis makes their study a field of major significance. Clearly PIN/LC8 plays a large role in the life of these unique and fascinating cells, and further study will help to solve the importance of this relationship.

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