ABSTRACT

SUNG, EUI JAE. The Role of Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) in Cell Motility. (Under the direction of Dr. Samuel L. Jones).

Recovery from inflammation requires that neutrophils migrate and infiltrate into the site of inflammation to clear the exogenous invaders and dead cell debris. Unfortunately, host cell damage is inescapable during overwhelming cell migration, tissue infiltration and bacterial killing. Thus, investigating the mechanism that regulates neutrophil adhesion and migration will provide us with insights to develop novel drugs to treat inflammatory diseases. One such target is myristoylated alanine-rich C-kinase substrate (MARCKS), a prominent protein kinase C (PKC) substrate. In most cell types, MARCKS is associated with the cell membrane in the resting state and is translocated into the cytosol when it is phosphorylated by PKC. This phosphorylation-dependent bilateral translocation of MARCKS is known to be involved in mucin- and neurosecretion, postnatal survival and regulation of cell morphology and migration. Previously, we have developed a cell permeable peptide named myristoylated N-terminal sequence (MANS) (corresponding to the first 24 amino acids of MARCKS) which has been shown to inhibit MARCKS function. Utilizing this unique peptide MANS, it has been shown that MARCKS plays a critical role in regulating human neutrophil function including β2 integrin dependent adhesion, chemotaxis, degranulation and pro-inflammatory cytokine secretion. However, the specific PKC isoforms responsible for phosphorylation dependent MARCKS function in neutrophils and aspects of MANS inhibition on MARCKS function have yet to be determined.

The purpose of this work was to elucidate the mechanism of MARCKS involvement in cell motility. Each of three chapters includes investigation of molecular mechanisms of
MARCKS physiology upon chemoattractant stimulation in neutrophils or fibroblasts.

Chapter II explores the requirement for the PKC δ isoform in MARCKS phosphorylation in human neutrophils through pharmacological assays using specific PKC isoform inhibitors. PKC δ specific inhibitor rottlerin reduced fMLF-induced phosphorylation of MARCKS in a dose dependent manner with an IC$_{50}$ value of 5.079 μM. Further, subcellular fractionation studies revealed that PKC δ is the only isoform that is activated in response to fMLF or PMA in human neutrophils. Chapter III is a comparative study on MARCKS function in canine cells. Intermediary (LTB4, PAF, and IL8) or end-target (C5a) chemoattractants significantly induced canine neutrophil adhesion and migration, and MANS pretreatment significantly inhibited these functions. Chapter IV examines an inhibitory role of MANS in MARCKS-dependent migration in NIH-3T3 fibroblasts. Cell migration as measured by a scratch wounding assay or PDGF-induced transwell migration assay was significantly attenuated with treatment of MANS peptide or expression of MANS protein in the cells. Genetic structure-function analysis revealed that MANS-mediated attenuation of NIH-3T3 cell migration does not require the presence of the myristic moiety on the amino-terminus. Furthermore, no difference in cell migration was observed in MARCKS knockdown cells with or without MANS treatment, which indicates MANS inhibitory effect is MARCKS specific. Taken together, we demonstrate a role for MARCKS in regulating the migration of neutrophils and fibroblasts and provide evidence that MARCKS is a potential therapeutic target for the treatment of diseases associated with neutrophil mediated inflammation.
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The Role of Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) in Cell Motility

by

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BIOGRAPHY

Eui Jae Sung was born in 1975 in Seoul, South Korea, lived with his older sister, Eun-Young and parents, Ki-Choon Sung and Kum-Sook Kim who soon began to describe him as “always troublesome.” His rebellious nature made it hard for him to listen well. In elementary school he began to excel in Tae Kwon Do which taught him some discipline. In fact, as a middle school student he was invited to be on the South Korean Demonstration Team which was allowed to travel around the United States at a time when overseas travel was limited for South Koreans. Also in middle school, Eui Jae became very interested in the sciences and mathematics. His high school days were spent preparing to enter university and he graduated from Myoung Ji High School in 1994.

Eui Jae then began his studies at the College of Veterinary Medicine (CVM) at Seoul National University (SNU) and added a year of high level math and science classes to his curriculum, graduating in 1999. He spent the next three years doing military service in the Republic of Korea Airforce as a veterinary officer treating military dogs and overseeing about 50 soldiers working with food inspection, an experience he found to be very valuable. Following military service, Eui Jae entered the masters program at Department of Veterinary Internal Medicine CVM, SNU. It was during this time that he met his future spouse, Nayoung, who was specializing in veterinary ophthalmology. After completing his masters Eui Jae was an internist with a private animal clinic until his and Nayoung’s move to Raleigh, NC in 2007.
Although at that time Eui Jae had little research experience, Dr. Sam Jones was kind enough to invite him to be a volunteer researcher in the Leukocyte Biology Laboratory, CVM at North Carolina State University. Dr. Jones took him under his wing and taught Eui Jae many techniques. Eui Jae struggled with his English and had trouble forming questions which forced him to look for solutions to challenges on his own, another valuable experience.

In the fall of 2008 Eui Jae began his PhD program in Comparative Biomedical Sciences investigating cell signaling in neutrophil motility still under the direction of Dr. Sam Jones. Eui Jae is looking forward to beginning his new position as a post-doc researcher in the Inositol Signaling Group at National Institute of Environmental Health Sciences (NIEHS). He and Nayoung will welcome their first child, a son, in early June 2013.
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CHAPTER 1

OVERVIEW OF THE ROLE OF MARCKS IN INFLAMMATORY PROCESS
A. Introduction

The inflammatory response is essential for host survival following bodily injury. It includes (A) an initial cellular-based reaction to the traumatic injury, (B) adjustment of the “battle field” in the vicinity of incident area, (C) attraction and orchestration of inflammatory cells that kill and remove microbes or damaged host cells, and finally (D) healing of the tissue. All steps of the inflammatory response are designed to maintain homeostasis within the host. These steps must be well balanced and regulated with great accuracy in order to avoid either excessive or insufficient inflammatory responses. This process is vital as inappropriate immune responses may lead to morbidity or shortened lifespan of the host. Excessive host responses can aggravate inflammation and often lead to devastating results, such as chronic inflammatory diseases. Insufficient responses produce immunodeficiency-like syndromes which may cause uncontrollable infections and cancer [1]. Thus, a more detailed understanding of the molecular mechanisms regulating the inflammatory response and maintenance of host homeostasis is important. This greater understanding will lead to identification of novel therapeutic targets which can be used to control the inflammatory processes, and therefore contribute significantly to the treatment of many serious inflammatory diseases.

One such potential target is myristoylated alanine-rich C-kinase substrate (MARCKS), a prominent protein kinase C (PKC) substrate. It has been suggested that MARCKS is involved in mucin secretion, neurosecretion, postnatal survival, and the regulation of cell morphology and migration. MARCKS can be found in different areas of the cell depending
on its phosphorylation status or association with other cellular elements. In most cell types, MARCKS is associated with the cell membrane in the resting state. When MARCKS is phosphorylated by activated PKC or associated with calmodulin (CaM, which is activated by increased intracellular calcium concentrations), MARCKS will translocate from the plasma membrane into the cytosol [2]. In the cytosol, MARCKS is dephosphorylated by specific protein phosphatases, such as protein phosphatase 2A (PP2A) which is stimulated by cGMP-dependent protein kinase (PKG) [3]. MARCKS is released from CaM when intracellular calcium concentrations return to normal levels. Both of these scenarios result in MARCKS returning to and re-associating with the plasma membrane. The reversible binding of MARCKS to the plasma membrane has been implicated in controlling cellular morphology and intracellular cell signaling via the binding of F-actin. The reversible association of MARCKS with the plasma membrane also promotes interaction between actin and actin binding proteins, as well as regulates phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) signals within the cell [2].

Recently, our lab has investigated the role of MARCKS in the regulation of inflammatory responses via disruption of MARCKS function in various leukocytes and fibroblast cells. Both of these cell types are considered pivotal players in the inflammatory and wound healing processes [4]. We have developed a cell permeate peptide, myristoylated N-terminal sequence (MANS), which corresponds to the first 24 amino acids of MARCKS and includes the myristoyl moiety found on the MARCKS protein. Treatment of cells with MANS has been shown to inhibit MARCKS physiology [3,5]. Utilizing this peptide, we have demonstrated that disruption of MARCKS function leads to attenuated neutrophil chemotaxis
and decreased β2-integrin dependent adhesion upon stimulation with a variety of chemoattractants. We have also found that MANS treatment decreases leukocyte degranulation and macrophage migration. These results suggest that the N-terminus of MARCKS modulates normal MARCKS function and is an important regulator in cells that are involved in inflammation. Furthermore, these results have encouraged us to investigate whether MANS can be used to treat serious inflammatory diseases involving leukocyte migration, adhesion and degranulation.

In this review, I will discuss MARCKS physiology and the methods investigators have used to unveil MARCKS function. In addition, I will elaborate on the ways in which MARCKS is involved with and coordinates surrounding factors to help regulate inflammation. I will do so by providing evidence that MARCKS affects and modulates cellular functions that are implicated in the key steps of the inflammatory process.

B. MARCKS Physiology

Myristoylated alanine-rich C kinase substrate (MARCKS) was first identified in 1982 as a substrate for calcium/phospholipid-dependent protein kinases in brain synaptosomes. Analysis of the protein by SDS-PAGE first suggested the protein was 87kDa in size [6], but was later revealed to be only 32 kDa, based upon calculations from its primary structure [7,8]. The overestimated molecular weight of MARCKS is due to special structural characteristics that it possesses, including a non-globular, natively unfolded form and a weak binding capability for SDS [2,9]. This unique protein, which is highly acidic and unusually rich in
alanine, has three distinct and highly conserved domains: an N-terminal myristoylated domain, a MARCKS 2 homology (MH2) domain, and a basic effector domain (ED) [8]. The N-terminus myristoyl moiety inserts hydrophobically into the lipid bilayer of the plasma membrane. The effector domain (ED), a 25-amino acid sequence that contains 13 basic amino acids, is capable of binding to the plasma membrane as well via its negatively charged phospholipids [10]. Together, these two mechanisms provide binding forces which secure MARCKS to the plasma membrane. The MH2 domain is a 15-amino acid residue which is identical to the sequence of the cytoplasmic tail of the mannose 6-phosphate receptor. The function of this domain is not yet clearly understood yet [11].

MARCKS interacts with the plasma membrane through its N-terminus myristoylated residue, and its ED. Myristoylation is a co-translational modification that involves the attachment of myristic acid, a common saturated fatty acid, to the amino group of the N-terminal glycine on MARCKS via an amide bond. The enzyme myristoyl-CoA: protein N-myristoyltransferase recognizes the specific sequence for myristoylation on the N-terminus of MARCKS and attaches the myristoyl chain to the N-terminal glycine residue [2]. This myristoyl chain is sufficiently hydrophobic and quickly becomes incorporated into the acyl core of the phospholipid membrane of the cell. This strong binding source is able to tether MARCKS to the cell membrane [12]. The ED, also known as the phosphorylation site domain, is a highly basic domain that is in charge of the majority of MARCKS’ interactions with ligands such as PKC, CaM, PIP2 and actin filaments [2,9,13,14]. The ED contains no acidic residues and interacts well with acidic lipids in the plasma membrane via electrostatic interactions [15-17]. In addition to highly basic characteristics, the ED also contains five
hydrophobic, aromatic phenylalanine residues. The hydrophobic nature of the ED allows insertion of the MARCKS ED between the lipid head groups found on the inner surface of the plasma membrane. This in turn provides another source of binding for MARCKS to the membrane of the cell [17,18].

The N-terminus myristoylated region and the ED both participate in tethering MARCKS to cellular membranes. Each one of these regions is important and necessary to sustain MARCKS binding to the plasma membrane. The critical role of these portions of MARCKS has often led researchers to target these regions in order to determine aspects of MARCKS physiology [19-22]. The cyclic, bi-directional translocation of MARCKS between the plasma membrane and the cytosol is a critical phenomenon which helps to regulate many cellular functions. These functions include embryonic/multi-organ development, neurosecretion, mucin secretion, endocytosis, exocytosis, phagocytosis, morphology modification and cellular migration. In the next section, I will review and summarize the methods that investigators use to work with and modify these two important regions that are critical for attachment of the protein to the membrane. This will give us a better understanding of MARCKS’ role in regulating and controlling the inflammatory process.

1. Study of MARCKS through the Myristoylated N-terminal Region

Myristoylation is required, but not completely sufficient, to anchor proteins to the cellular membranes [20,23]. For the most part, membrane association of myristoylated proteins requires an additional signal to sustain the association. For MARCKS, the
additional signal is provided by the electrostatic force of the ED. To determine the importance of the role of the myristoylated region of MARCKS, researchers have modified this region by either, 1) replacing the myristic acid with a moiety that has a stronger affinity for the plasma membrane, or 2) deleting the myristoyl moiety altogether. Myat et al. suggested that strong attachment of MARCKS to the plasma membrane affects cell spreading and membrane ruffling [24]. To investigate this, they replaced the single myristoyl moiety on the N-terminus of MARCKS with two palmitoyl residues, creating a stronger attachment of the protein to the plasma membrane. Expression of this palmitoyl mutant MARCKS in fibroblast cells resulted in arrest of cell spreading, membrane ruffle formation, and cell adhesion to fibronectin. In contrast, deleting the myristoyl moiety from the MARCKS protein in myoblast cells led to more rapid cell spreading when compared to wild type MARCKS [25]. Deletion of the myristoyl moiety was accomplished via mutation of the first amino acid of the N-terminus of MARCKS from a glycine (G) to an alanine (A), effectively eliminating the myristoylation domain. This study found that the majority of mutant MARCKS was located in the cytosolic fraction of the cells within 1 hour after plating. However, within 4 hours of cell adherence to a fibronectin coated plate, the mutant MARCKS had returned to the membrane fraction of the cell. This study also found that these cells containing the mutated MARCKS displayed a normal cell spreading pattern. These results suggest the possibility that myristoylation of MARCKS protein is not essential for the shuttling of MARCKS between the membrane and the cytosol, and therefore for normal protein function. Similarly, Swierczynski et al. reported that myristoylation of MARCKS is not necessary for correction of developmental abnormalities seen in cases of MARCKS protein deficiency [26].
It has been shown that MARCKS deficiency causes universal perinatal casualties including defects in neurulation, fusion of the cerebral hemispheres, formation of the great forebrain commissures, and retinal and cortical lamination in mice [27]. Expression of unmyristoylated MARCKS in MARCKS-deficient mice resulted in rescuing of the normal phenotype and ablation of the anatomical defects associated with MARCKS deficiency in both survivors and nonsurvivors. However, the survival rate of mice in this study was only 25% [22]. These results indicate that myristoylation of MARCKS is critical for the protein to function to its full extent, while unmyristoylated MARCKS can still serve as a regulator of cell spreading and organelle development.

Li et al. have demonstrated a tight relationship between mucin secretion and MARCKS physiology, especially through the myristoylated N-terminus [3]. They found that treatment of cells with a peptide consisting of the first 24 amino acid of MARCKS, including an N-terminal myristoylated moiety, results in significant inhibition of mucin secretion in normal human bronchial epithelial cells (NHBEs). The peptide was named MANS (myristoylated N-terminal sequence), and subsequent experiments utilizing MANS revealed that intratracheal instillation of the peptide blocks mucin hypersecretion in a mouse model of asthma [5,28]. A missense peptide called RNS (random N-terminal sequence), which contains the same amino acid composition as MANS arranged in random order, has no effect on mucin hypersecretion. Singer et al. showed that hypersecretion of mucin in ovalbumin (OVA)-sensitized, methacholin or pilocarpine-inhaled mice is significantly attenuated after intratracheal or intranasal application of MANS. In addition, they reported that MANS treatment causes interference of MARCKS association with mucin granule membranes [5].
Agrawal et al. further revealed that MANS therapy relieves airflow obstruction caused by allergenic mucin hypersecretion [28]. These results suggest that MARCKS plays an important role in regulating mucin hypersecretion, and that the MANS peptide can potentially treat serious obstructive respiratory diseases via interference with MARCKS physiology.

The development of MANS has brought further interest to investigation of the N-terminal region of MARCKS and its involvement in regulation of cellular secretion and motility. Initial applications of the MANS peptide have been targeted towards leukocytes involved in obstructive respiratory diseases, such as COPD (Chronic Obstructive Pulmonary Disease). COPD is an inflammatory disease in which leukocytes are a key player, due to the migration of these cells to the respiratory tract and release of inflammatory mediators that tend to exacerbate the condition [29]. Furthermore, MARCKS is the one of the most substantial proteins in leukocytes [9]. Takashi et al. showed that MANS treatment of neutrophils, eosinophil-like cells, monocyte-like cells and natural killer (NK) cells results in attenuated myeloperoxidase, eosinophil peroxidase, lysozyme and granzyme C release, respectively [30]. All of these processes have been shown cause severe damage to tissues during the pathogenesis of inflammatory disorders and therefore warrant therapeutic consideration. Subsequent experiments have shown that MANS treatment of neutrophils from humans [4], dogs (Sung unpublished), and horses (Sheats unpublished) causes significant reduction in chemotaxis towards various chemoattractant stimuli as well as decreased β2-integrin dependent adhesion. Further, studies have shown that mouse macrophages undergo decreased chemotaxis when cells were treated with the MANS peptide
These results reveal a role for the myristoylated N-terminus in the regulation of leukocyte degranulation, adhesion, and migration. Indeed, further investigation should be conducted to unveil the specific role of MANS in the regulation of the cells. These results suggest that MARCKS is a potential target for the development of novel anti-inflammatory medications for treating serious inflammatory diseases in many species, including humans.

2. Study of MARCKS through the Effector Domain (ED)

Another important MARCKS protein domain that is capable of associating with the plasma membrane is the effector domain (ED) or phosphorylation site domain (PSD). The ED consists of 13 basic lysine and arginine residues, five hydrophobic phenylalanine residues and four serine residues; this composition causes this region to be highly basic and enables it to electrostatically interact with acidic phospholipid bilayer. Also, the five aromatic phenylalanines of MARCKS can insert themselves into the lipid head-group region and provide an additional source of binding to the plasma membrane [2]. The ED also serves as a site for phosphorylation by PKC, specifically at three of the four serine residues found on murine MARCKS: Ser 152, Ser156, and Ser 163 [32]. Binding motifs for calcium concentration dependent calmodulin (CaM) and actin are also found within the MARCKS ED. An additional feature of the MARCKS ED is found in the polylysine region, which binds to and sequesters a minor lipid component of the plasma membrane, phosphatidylinositol 4,5-bisphosphate (PIP2). PIP2 controls the activity of numerous proteins and is a source of second messengers within the cell. MARCKS tethering to PIP2
rich areas on the plasma membrane helps to regulate PIP2 and therefore second messenger availability [33-35]. The importance of this function has driven many researchers to investigate MARCKS function via study of the ED.

MARCKS phosphorylation is particularly important for MARCKS function within the cell. In a resting state, the positively charged MARCKS ED interacts with the negatively charged plasma membrane resulting in MARCKS membrane association. Phosphorylation, which includes the addition of negatively charged phosphate groups to serine residues on the ED, neutralizes the positive charge of the ED and abrogates MARCKS interaction with the plasma membrane. This results in the dissociation of MARCKS from the plasma membrane and translocation to the cytosol [13,36]. In addition, MARCKS phosphorylation has been shown to significantly decrease the affinity of MARCKS for actin or CaM [11]. Thus, phosphorylation is a crucial regulator of MARCKS function within the cell.

To study MARCKS activity, researchers have modified the phosphorylation status of the MARCKS via manipulation of serine residues found in the ED [25,37,38]. In doing so, researchers have generated both a phosphorylation-resistant MARCKS mutant, as well as a pseudo-phosphorylated MARCKS mutant. A phosphorylation-resistant MARCKS mutant was created via substitution of four serine residues in the ED with alanine (A), glycine (G), or asparagine (N), all of which have isoelectric points similar to serine. A pseudo-phosphorylated ED, was created via substitution of the same four serine residues of the ED with aspartic acid (D), which emulates the presence of a phosphate. Using these methods, researchers have demonstrated that phosphorylation modifications of MARCKS affect MARCKS translocation within the cell. Pseudo-phosphorylated MARCKS has been found to
be localized to cytosolic compartments in mouse melanoma cells [38] and in human embryonic kidney (HEK) 293 cells [37]. In contrast, both wild-type and the non-phosphorylatable mutant MARCKS protein exhibit predominantly plasma membrane localization. Interestingly, the localization of MARCKS does not always correlate with the ability of MARCKS to regulate cell function. For example, both non-phosphorylatable and pseudo-phosphorylated mutants interfere with the adhesion of HEK 293 cells, although mutants are localized differently within the cell [37]. In contrast, the pseudo-phosphorylated mutant has been shown to have a pronounced effect on the movement of weakly motile F1 mouse melanoma cells, whereas non-phosphorylatable mutant has no effect on the cells at all [38]. These opposing results suggest that MARCKS appears to function differentially in different cell types. The precise ways in which MARCKS regulates cell motility and adhesion remains to be determined, as well as which aspects of MARCKS cause these cell specific differences.

C. The Inflammatory Process

Inflammation is a natural defense mechanism against both internal and external agents that trigger bodily injury, as well as an essential apparatus in the process of healing. It is impossible for a host to survive without diligent sentinels and their sincere, vigilant guard. Sentinel cells including macrophages, mast cells, and dendritic cells continuously survey the body for the presence of danger. Inflammation begins when these sentinel cells detect danger signals, such as invasion of foreign antigens or dead host cell debris. They begin to process
these signals and transfer them to neighboring epithelial cells, endothelial cells or fibroblasts, as well as appropriate immune effector cells such as neutrophils, T cells and B cells. Non-immune cells (e.g., epithelial cells) in the area release chemokines and chemoattractants to recruit immune cells to the “battlefield.” The recruited immune cells are activated via the chemokines, chemoattractants, and transferred danger signals, and are then ready to fight the foreign agent. Once on the battlefield, immune cells use various types of weapons to kill the dangerous endogenous or exogenous agents. After the danger has been eliminated, the affected area begins the healing process. Tissue healing requires infiltration of many cell types, including leukocytes, which provide scaffolds for newly forming granulation tissue. This granulation tissue consists of various cells, such as endothelial cells, fibroblasts, and macrophages, all of which help to restore tissue integrity [39,40].

As was just described, the inflammatory process is heavily based upon the involvement and orchestration of various cells types. Recent studies by our lab as well as others suggest that MARCKS is an important regulator of inflammatory cells, such as neutrophils, macrophages, fibroblasts, endothelial cells, and lymphocytes. MARCKS has been shown to mediate a variety of cellular functions, including cell morphology, migration, adhesion, secretion, endo/exocytosis, and phagocytosis. Merging our knowledge of MARCKS function with its role in inflammatory cells creates a better understanding of MARCKS as a potential therapeutic target in inflammatory disease. It should be noted that there are a multitude of events that can cause inflammation, and each could lead to a different consequence involving MARCKS. Therefore for simplicity, the following review will only cover mild trauma accompanied by mild bacterial infection.
1. Involvement of MARCKS in response to early danger signals

When tissue damage or injury occurs, a variety of responses are produced in an effort to protect the host from harm. First, cells in the area of injury will release endogenous danger signals, called damage-associated molecular patterns (DAMP). Such signals include heat-shock proteins (HSP), high-mobility group box 1 (HMGB1), mitochondrial peptides containing N-formyl group, and cytokines such as IL-1α and IL-33. These signals are able to trigger cytokine production and initiate the inflammatory processes in the area of insult [1,41-46]. Pain is a result of traumatic injury and released intracellular proteins, and leads to neuronal secretion of bioactive peptides [47]. In addition, bacterial penetration and the secretion of bacterial products occur in the area of injury. Bleeding may also occur as a result of disruption of blood vessels.

Early alert signals produced by dead and necrotic cells are recognized by specialized cells within the body. These specialized cells are capable of releasing mediators required for initiating inflammatory responses. One of these specialized cells types is the mast cell [1]. Mast cells are located at bodily sites that are routinely exposed to the external environment [48,49]. They are designed to quickly release various signaling molecules upon stimulation, such as histamine, proteases, chemokines, and cytokines [50]. Mast cells have been shown to express MARCKS protein, and stimulation of these cells with PMA results in phosphorylation and translocation of MARCKS from the plasma membrane to the cytosol [51]. A study performed in 1993 by Ozawa et al. provided evidence that both PKC β and δ isoforms are involved in mast cell degranulation [52]. A later study revealed that PKC β1
specifically is an important regulator of degranulation in mast cells [53]. At that time, the role of MARCKS in mast cell degranulation was not well understood. Recently, Gadi et al. suggested that MARCKS may be involved in mast cell degranulation via a PKC βI dependent pathway. They found that the oscillatory association of PKC βI with the plasma membrane in mast cells is synchronized with oscillations in intracellular Ca\(^{2+}\) concentrations. This phenomenon is also found to coincide with the reversible, oscillatory dissociation of the MARCKS-ED with the plasma membrane under stimulatory conditions. In addition, this study demonstrated that expression of a non-phosphorylatable MARCKS ED mutant in mast cells results in delayed Ca\(^{2+}\) mobilization and degranulation [54]. While these results suggest a role for MARCKS in mast cell degranulation, it should be noted that MARCKS involvement in mast cell function may not be the same across all mast cell populations. One group has reported that MARCKS is differentially expressed in mast cell depending on their origin [55]. MARCKS is up-regulated in neonatal cord blood (CB)-derived mast cells, but not in peripheral blood (PB)-derived mast cells. These results suggest a differing role for MARCKS in regulation of degranulation in mast cells derived from neonatal hematopoietic cells when compared to their adult counterparts.

The nervous system also regulates inflammatory responses in an unconscious, prompt, and effective manner [56]. The concept of inflammatory stimuli activating anti-inflammatory signals was first introduced in the 1950s [57]. It is now known that neuronal cells produce and release a variety of inflammatory regulators, such as TNF and IL-1, which also participate in neuronal communication [58, 59]. MARCKS is widely expressed in the CNS and localizes in axons, axon terminals, and small dendritic branches [60]. MARCKS has
been implicated in synaptic trafficking, neurotransmitter release, and neuronal exocytosis [2,61-63]. Rose et al. showed that MARCKS phosphorylation leads to F-actin disassembly associated with vesicle recruitment to the plasma membrane, as well as participates in catecholamine release in chromaffin cells [64]. The exocytosis of neurotransmitter-containing large dense-core vesicles (LDCVs) has also been found to depend heavily on phosphorylation and activation of MARCKS via PKC isoform ε [65]. Tatsumi et al. used formalin injection tests to demonstrate that MARCKS is involved in inflammatory pain and the maintenance of neuropathic pain [66]. These results suggest that MARCKS plays an important role in the inflammatory process via the nervous system.

Early alert signals, including exogenous signals (bacteria and their products: LPS, formyl-peptides), endogenous signals (DAMP, HSP, HMGB1, and cytokines), as well as neurogenic peptides, can cause activation of resident macrophages. Activated macrophages secrete chemoattractants that result in the recruitment of leukocytes to an injured site [1]. Macrophages express a substantial amount of MARCKS protein [67], which is localized in the plasma membrane and podosomes of these cells [68]. To investigate the role of MARCKS in macrophages, Carballo et al. collected and observed fetal liver-derived macrophages from MARCKS knockout mouse embryos. This study found that MARCKS-deficient macrophages have almost the same morphology, phagocytic function, and macropinocytic function as wild-type macrophages. The only exception is a significant and reproducible decrease in zymosan phagocytosis found in the MARCKS-deficient macrophages [69]. A concurrent study performed by Underhill et al. demonstrated that a protein similar to MARCKS, MacMARCKS (also known as MARCKS-related protein), is
not necessary for phagocytosis, although it does associate with phagosomes [70].

Interference with MARCKS protein function in J774A.1 cells (mouse macrophage cell lines) and primary mouse macrophages via treatment with MANS peptide causes attenuated migration towards chemoattractants MCP-1 and C5a [31]. Furthermore, treatment of primary human macrophages with MANS or its structural analog BIO-11000 [71][72], causes significant inhibition of secretion of macrophage-specific apolipoprotein (apo) E, a regulator of atherosclerosis [72]. This suggests that MARCKS is an important regulator of macrophage exocytosis. Together, these results indicate that MARCKS plays a critical role in innate immunity via regulating secretion and migration of macrophages. Additionally, MARCKS does not appear to have a prominent effect on phagocytosis in macrophages, but may still play a small part in this activity, at least in the phagocytosis of zymosan by these cells.

Tissue injury commonly causes the disruption of blood vessels and results in hemorrhage. Maintenance of hemostasis is required at this time to prevent blood loss and maintain adequate blood flow within vessels. Platelets are the first cells to reach exposed subintimal structures and they play a prominent role in maintaining normal hemostasis [40,73]. Platelets require activation, accompanied by shape change, pseudopodia formation, aggregation, and secretion to properly fulfill their function [74-77]. Activation of platelets is PKC-dependent [78-82] and MARCKS plays a central role in this process [83]. Elzagallaa showed that platelets expresses MARCKS protein, and that PMA-induces MARCKS phosphorylation correlated with serotonin release from permeablilized platelets in a time-dependent manner. The secretion of serotonin is blocked by treatment of platelets with a
peptide identical in structure to the MARCKS-ED [83]. These results provide evidence that MARCKS plays an important role in platelet hemostatic activities.

The studies presented above support the idea that MARCKS induces the initial steps of the inflammatory process by, 1) regulating sentinel cells such as mast cells and macrophages, 2) controlling neuronal secretion, and 3) maintaining hemostasis. If no significant issues arise at this phase, the inflammatory process proceeds to the next step.

2. Adjustment of the Battlefield in the Area of the Incident

Various types of mediators, including histamine, bradykinin, platelet activating factor (PAF), eicosanoids, proteases and TNF, are secreted by sentinel cells and platelets to promote dilatation of small blood vessels near sites of injury [40]. Vasodilation results in increased blood flow to the area, which leads to the development of hyperemia. Inflammatory mediators also stimulate endothelial cells and cause increased vascular permeability. Increased vascular permeability bolsters leukocyte transmigration from the bloodstream to the incident area and leads to accumulation of exudates [1]. Protein-rich exudate is beneficial to the host for many reasons, including: 1) dilution of harmful substances, 2) delivery of leukocytes to the area to help remove bacteria and cell debris, and 3) delivery of clotting proteins that help to form a fibrin mesh in the incident area, effectively to isolating and prevent the spread of bacteria and other toxic agent into adjacent tissues or the bloodstream [1,56,84].
It has been suggested that MARCKS is substantially involved in regulation of endothelial permeability. Jacobson et al. have shown that both thrombin and histamine stimulate MARCKS phosphorylation in human umbilical vein endothelial cells (HUVECs) [85]. They also found that treatment of these cells with thrombin receptor residue peptide (TR; thrombin receptor) causes phosphorylation of MARCKS as well. This indicates that thrombin induces MARCKS phosphorylation in HUVECs via thrombin receptors, also known as protease-activated receptors (PARs). Histamine also causes phosphorylation of MARCKS, an event which can be blocked by treatment with the H<sub>1</sub>-blocker pyrilamine, but not by the H<sub>2</sub>-blocker cimetidine. This suggests that histamine-induced MARCKS phosphorylation is via an H<sub>1</sub>-type receptor mechanism. Both thrombin- and histamine-induced MARCKS phosphorylation in HUVECs can be blocked by staurosporine treatment, which suggests that both of these signals are PKC-dependent [85]. The finding that thrombin and histamine alter endothelial permeability in HUVECs [86] and MARCKS-dependent degranulation in mast cells [54] suggests that MARCKS plays a central role in regulating endothelial cell permeability throughout early stages of the inflammatory response.

In addition to thrombin and histamine, insulin [87] and hydrogen peroxide [88] have also been shown to regulate vascular endothelial physiology via a MARCKS-dependent mechanism. Kalwa et al. found that MARCKS co-localizes with caveolin-1 in plasmalemmal caveolae, which serve as sites for sequestration of PIP2. This association is disrupted by insulin-induced MARCKS phosphorylation in bovine aortic endothelial cells (BAECs). Additionally, MARCKS knockdown using siRNA attenuates directed movement of endothelial cells, blocks insulin-induced PIP2 accumulation in plasmalemmal caveolae, and
blocks phosphorylation of N-WASP and Arp2/3 (PIP2-binding and actin-binding proteins, respectively) [87]. Similarly, Jin et al. showed that hydrogen peroxide increased endothelial permeability in BAECs and that this mechanism could be significantly blocked by siRNA MARCKS knockdown in these cells. They also revealed that H$_2$O$_2$-induced endothelial alterations are MARCKS phosphorylation-dependent via activation of the Rac1-Abl1-Phospholipase C$\gamma$1-PKC$\delta$ loop [88]. These findings suggest that MARCKS is a critical mediator of endothelial permeability via various mechanisms that affect the architecture of the actin cytoskeleton, including PKC signals and PIP2 metabolism.

3. The Involvement of MARCKS in Immune Cells

Leukocytes such as neutrophils, monocytes, and lymphocytes, infiltrate sites of injury in response to chemoattractants released from activated endothelial cells, aggregated platelets, and sentinel cells [40]. They are effector cells whose job is to fight against exogenous pathogens as well as remove debris and regenerate tissue. Among the different leukocytes, neutrophils are the most prominent cells because, 1) they are the first to arrive in affected areas, 2) they arrive in great numbers, and 3) they possess hydrolytic, oxidative, and pore-forming weapons to kill bacteria [89].

In order to reach the area of injury, neutrophils must emigrate from the blood stream into the tissue and then migrate to the site of inflammation. Interaction of binding molecules called integrins on the neutrophil cell surface with their ligands on the endothelium results in adherence of circulating neutrophils to the vascular endothelium. This is followed by
transmigration of the neutrophils through the endothelial cells and into the tissues. Integrins also play a critical role in neutrophil chemotaxis and adhesion-dependent neutrophil functions, including oxidative burst and phagocytic clearance of pathogens [90,91]. There are some studies that show that MARCKS is implicated in neutrophil functions in a variety of contexts [4,9,30,36,71,92-94], and it has been revealed that MARCKS is an important regulator of neutrophil adhesion and migration [4]. MARCKS is phosphorylated upon stimulation with the chemoattractant fMLF and the PKC activator PMA [36]. Our lab has demonstrated that fMLF-induced phosphorylation is PKC δ dependent in human neutrophils (Sung et al. unpublished). We have also found that MANS peptide pretreatment of human neutrophils causes dislocation of MARCKS from the plasma membrane [4]. In addition, we have shown that MANS pretreatment results in significant reduction of chemoattractant-induced migration and β2-dependent adhesion of neutrophils from humans [4], dogs (Sung et al. unpublished) and horses (Sheats et al. unpublished). In addition to adhesion and migration, MARCKS has been shown to be involved in degranulation and cytokine release in neutrophils. MANS treatment attenuates PMA-induced myeloperoxidase secretion [30] and LPS-induced protein expression of IL8 and TNFα [94] in neutrophils. These findings strongly suggest a critical role of MARCKS in regulating neutrophil functions.

The innate and adaptive immune systems must cooperate with one other in order to protect the host against exogenous pathogens. Classical concepts of immunity dictate that innate immune cells (including neutrophils and macrophages) are the first cells to arrive on the scene to help control infection in the initial phases of the immune response. Dendritic cells and other antigen presenting cells are the next cells to arrive, and they act by presenting
processed antigens to helper T cells to induce the adaptive immune system. T cells are responsible for cell-mediated immunity, and B cells are in charge of humoral responses. Until recently, many have believed that both of these responses usually take several days or even weeks to develop. However, recent findings indicate that T cells [95] and B cells [96] are actually important participants in acute and early inflammatory responses as well. Kim et al. showed that T cell or lymphocyte-deficient mice with viral infections or injected with polyinosinic-polycytidylic acid (Poly I:C, a ligand for TLR3) had higher early mortality rates compared with wild-type mice. Further, studies involving depletion of CD4+ and CD8+ cells in wild-type mice and the transfer of T lymphocytes into Rag-1-deficient mice, demonstrated that T cells are both necessary and sufficient to decrease early innate responses, including the levels of pro-inflammatory cytokines (such as TNF and INF-γ) produced during this phase [95]. Similar to these findings, Kelly-Scumpia demonstrated that B cell-deficient or anti-CD20 B cell-depleted mice had reduced survival rates and levels of serum innate inflammatory cytokines such as IL6, IL1β, IL10, INF-γ, and MCP-1. This study also found that transferring B cells into Rag-1-deficient mice caused these mice to displayed enhanced sepsis survival [96]. These results indicate that B cells have a protective role in early innate immune responses during bacterial sepsis.

Studies of MARCKS involvement in lymphocyte function are scarce, but an early report from 1989 demonstrated that MARCKS is differentially regulated during T- and B-cell development and activation [97]. This study showed that MARCKS was expressed at a higher level in B cells than in T cells, and that the expression of MARCKS in B cells was greatly enhanced by antibody stimulation. However, it was found that PKC activation of
lymph node T cells or of T cell hybridomas did not induced phosphorylation of MARCKS [98] or the expression of MARCKS protein [97],[98]. The same study revealed that MARCKS was expressed at high levels in immature thymocytes and at low levels in mature thymocytes. This indicates that MARCKS is differentially expressed in T cells depending on their developmental stage. In seeming contrast to these findings, Smeets et al. demonstrated that PMA stimulation of Jurkat T cells did result in MARCKS phosphorylation [99]. Although they have a significant number of signaling pathways in common, this finding suggests that Jurkat cells and primary T cells display differential gene expression patterns; a conclusion that is supported by comparative microarray analysis [100]. This discrepancy should be considered when investigating MARCKS-dependent signaling in T cells using Jurkat cells.

In contrast to T cells, mature B cells constitutively express MARCKS, and stimulation of their antigen receptors using anti-immunoglobulin increases the expression of MARCKS protein in B cell blasts [97]. A recent case report suggests a strong relationship between B cell-related immunodeficiency syndrome and MARCKS function [101]. Salzer et al studied a patient who suffers from common variable immunodeficiency (CVID)-like primary B cell immunodeficiencies (B-PID) with progressive B cell lymphopenia. Using whole blood and peripheral blood mononuclear cells (PBMC) from this patient, they uncovered a biallelic splice-site mutation in the gene encoding PKC δ, *PRKCD*. In addition, they showed that this patient had an absence of PKC δ and significantly reduced expression of MARCKS and phospho MARCKS compared to healthy individuals. These findings suggest that MARCKS may be implicated in B cell-related immunodeficiency.
4. MARCKS Involvement in the Resolution of Inflammation

The healing process is very dynamic. It involves complex crosstalk between signaling molecules and various cell types, including infiltrated leukocytes, macrophages, keratinocytes, fibroblasts, and endothelial cells [39,40]. Antibacterial or tissue damaging reactions, including infiltration of leukocytes and secretion of inflammatory mediators, initiate proliferative responses for tissue repair and epithelial closure [1]. During proliferative phases, injured tissue or wound space is covered or filled by newly formed epithelium and granulation tissue. Growth of new blood vessels, or angiogenesis, also occurs during this time. After new tissue is formed, tissue remodeling begins in an effort to restore normal tissue structure and function [39,40]. Keratinocytes, fibroblasts, and vascular endothelial cells are the dominant cell types that participate in the resolution phases.

Migration and proliferation of keratinocytes is necessary for restoring intact epidermis in a damaged area. Several elements are involved in keratinocyte migration, including extracellular matrix (ECM) collagens and fibronectin, matrix metalloproteinases (MMPs), and growth factors such as epithelial growth factor (EGF) and transforming growth factor (TGF) [102]. Keratinocytes also utilize integrins on their surface to migrate on specific substratum, especially α2β1 integrins for type I collagen [103] and α5β1 for fibronectin [104]. Blocking these integrins results in dramatic inhibition of keratinocyte migration [103,105].

EGF and TGF-α have been shown to be important stimuli for keratinocyte migration, proliferation, and epithelialization [40], as well as critical mitogens for keratinocytes and
fibroblasts [106-108]. Reynold et. al showed that EGF and TGF-α induce mild or decreased MARCKS phosphorylation in keratinocytes in the absence of DAG formation, when compared to fibroblasts [109]. Subsequent studies demonstrated that raising intracellular Ca$^{2+}$ concentration in the presence of EGF induces keratinocyte differentiation and stimulates PKC activity without inducing MARCKS phosphorylation [110,111]. However, treatment of cells with a calmodulin inhibitor, calmidazolium, rescued MARCKS phosphorylation in a dose-dependent manner via Ca$^{2+}$-mediated PKC activation [111]. These findings suggest that MARCKS may affect EGF- or TGF-α-induced keratinocyte function via Ca$^{2+}$-mediated calmodulin signaling, instead of through PKC-dependent pathways.

Granulation tissue formation is also needed for tissue reconstruction [40]. Many different elements are involved in the formation of granulation tissue, including, 1) fibroplasia, the accumulation and proliferation of fibroblasts in the incident area, and 2) angiogenesis, which includes endothelial cell activation, proliferation, and eventually the formation of new vasculature at the wound site.

Fibroblasts are the major cell type responsible for synthesizing and remodeling ECM in tissue. They produce and respond to growth factors, which regulate development, differentiation, and repair of many tissues [112]. Furthermore, they release cytokines and chemokines to attract and activate immune cells from lymphoid tissues including the thymus, spleen, and lymphnodes [113,114]. Fibroblasts also induce inflammatory infiltration in early stages of inflammation [115]. They contribute to the resolution of inflammation by stimulating apoptotic signals in leukocytes or normalizing chemokine gradients to stop
immune cells from infiltrating the tissue in later stage of the inflammatory response [116]. Therefore, fibroblasts are an attractive target for anti-inflammatory therapy [117].

Studies regarding MARCKS function in fibroblasts are abundant and began relatively early in the 1990s. One such study found that MARCKS expression levels can be altered by transformation of oncoprotein c-ras or v-src into NIH 3T3 fibroblasts [118], suggesting that MARCKS may be involved in the development or maintenance of transformed characteristics. Peptide mitogen bombesin [119], bradykinin, vasopressin, and PMA [120] have been found to induce MARCKS phosphorylation in fibroblasts via PLC-PKC dependent pathways. Pretreatment of cells with the PKC inhibitor staurosporine suppresses PMA-induced MARCKS phosphorylation [121]. Allen et al. demonstrated that MARCKS translocation between the plasma membrane and Lamp-1 positive lysosomes in the cytosol is a PKC dependent process in fibroblasts [122]. Uberall et al. showed that PKC isotypes α, ε, and θ are responsible for MARCKS phosphorylation in these cells [123]. Fibroblasts derived from MARCKS knockout mouse embryos revealed that MARCKS does not appear to participate in endocytosis or pinocytosis [69], but does affect the morphology and motility of fibroblasts. It was demonstrated that MARCKS regulates membrane ruffling and cell spreading in fibroblasts via bilateral translocation between the plasma membrane and the cytosol [24]. Furthermore, EGF-induced MARCKS phosphorylation has been implicated in fibroblast contractility and motility through a PKC isoform δ dependent pathway [124]. Recent experiments performed by Ott and Sung demonstrated that MARCKS regulates fibroblast migration through its N-terminus region, and myristoylation does not seem to be involved in this migration (Ott et al, under review). Thus, these results suggest that
MARCKS is a critical regulator of fibroblast functions, especially those involving morphologic modification and migration.

As stated above, MARCKS is expressed in endothelial cells and is involved in vascular permeability. With this regard, several reports have suggested that MARCKS is also implicated in angiogenesis in a PKC-dependent manner. It has been reported that PMA-induced PKC activation results in the induction of angiogenesis in vitro and in vivo [125-127]. However, PKC isoforms display differential effects on endothelial cells. Murakami et al. showed overexpression of PKC δ causes a reduction of endothelial cell differentiation [128], but Wang et al. demonstrated that inhibition of PKC α attenuates vascular formation in vitro and in vivo [129]. Xu et al. reported that vascular endothelial growth factor (VEGF) induces MARCKS phosphorylation via a PKC α-dependent pathway and improves angiogenic activity in HUVECs [130]. Zhao et al. observed that during endothelial proliferation in bovine pulmonary microvascular endothelial cells (BPMECs), MARCKS protein levels are downregulated [131], suggesting that MARCKS has a regulatory role in angiogenesis. Together, these results suggest that MARCKS is involved in endothelial cell function, and thus contributes to angiogenesis through PKC-dependent pathways.

D. Conclusion

Dysregulation at any step of inflammatory process may prove to be more troublesome than the original inciting stimulus. An overactive or underactive immune response may interfere with host defensive mechanisms and hinder the complex process of infection
Diverse populations of cells participate in immunity and must perform their functions properly in order to pricelessly regulate the strength of the inflammatory response. In the majority of these cells, such as mast cells, macrophages, endothelial cells, leukocytes, and fibroblasts, MARCKS plays a mediating role as a either a signaling inducer or a negative regulator. This role is essential to establishing a perfectly coordinated immune response. Thus, a more thorough understanding of MARCKS physiology and expression in the appropriate cells is beneficial to avoiding dysregulation of inflammatory responses. Although extensive research has been performed, the precise mechanisms of MARCKS in the inflammatory process are still unclear. Therefore, further research is necessary to elucidate the roles of MARCKS in specific inflammatory process.
REFERENCES


CHAPTER 2

MARCKS PHOSPHORYLATION RELIES ON PKC ISOFORM DELTA (δ) IN HUMAN NEUTROPHILS

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Abstract

Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) is a Protein Kinase C (PKC) substrate that is involved in mucin- and neurosecretion, postnatal survival and regulation of cell morphology and migration. In most cell types, MARCKS is associated with the cell membrane in the resting state and is translocated into the cytosol when it is phosphorylated by PKC. We previously determined that MARCKS plays an important role in neutrophil migration and adhesion using a cell permeant peptide derived from the MARCKS myristoylated aminoterminus (MANS). To unravel how MARCKS phosphorylation affects neutrophil migration and adhesion, we identified the PKC isoforms involved in MARCKS phosphorylation. We tested PKC isotypes alpha (α), beta (β), delta (δ), and zeta (ζ), known to be present in human neutrophils, using specific PKC isotype inhibitors Go6976, CG53353, rottlerin and ζ pseudosubstrate, respectively. Human neutrophils were pretreated with inhibitors and MARCKS phosphorylation was measured after stimulation of cells with fMLF. Pretreatment of neutrophils with the pan-PKC inhibitor staurosporine blocked fMLF induced MARCKS phosphorylation in a dose dependent manner, as did specific inhibition of PKC delta; but other PKC isoforms including alpha, beta and zeta did not affect MARCKS phosphorylation. The IC50 of staurosporine and rottlerin were 1.094 μM and 5.709 μM respectively for the fMLF induced MARCKS phosphorylation. Subcellular fractionation assay showed that the PKC isotype delta, but not others, translocated from cytosol to membrane in response to fMLF and PMA stimulation. These results suggest that PKC delta plays a crucial role in fMLF induced MARCKS phosphorylation in human neutrophils.
Introduction

Neutrophils play a primary role in host defense since migration of neutrophils to sites of infection or inflammation is critical for pathogen clearance and tissue repair [1,2]. Infiltration of neutrophils in the incident area is essential for host defense against infectious agents; however, massive neutrophilic inflammation can also be deleterious, leading to systemic or chronic inflammatory disease [1]. A more detailed understanding of the molecular mechanism involved in neutrophil adhesion and migration could lead to the development of therapeutic drugs that lessen the tissue damage from neutrophil-related disorders.

Neutrophils are activated by a range of stimuli including chemoattractants, bacterial peptides, and inflammatory cytokines through their surface receptors [3,4]. The receptor-mediated intracellular signals regulate various neutrophil functions including migration, adhesion, phagocytosis, degranulation and respiratory burst [5-8]. One such signaling target of interest is Myristoylated Alanine-Rich C Kinase Substrate (MARCKS), a major protein kinase C (PKC) substrate.

MARCKS is an acidic, rod-shaped actin cross-linking protein that is associated with the plasma membrane via its hydrophobic N-terminus myristoylated domain and highly basic effector domain (ED) [9]. One of the interesting characteristics of MARCKS is its phosphorylation-dependent translocation pattern in the cell [10]. In most cell types, MARCKS is associated with the cell membrane in the resting state and is translocated into the cytosol when it is phosphorylated by activated protein kinase C (PKC). In the cytosol, MARCKS is dephosphorylated by a specific protein phosphatase such as protein phosphatase
2A (PP2A) that is stimulated by cGMP-dependent protein kinase (PKG) [11], which results in MARCKS re-association with the plasma membrane. This bilateral translocation of MARCKS is known to control cell morphology and intracellular cell signaling through binding F-actin, promoting the interaction between actin and actin binding proteins, and regulating phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) signals [9,12-16].

Previously, we have developed a cell permeable peptide, named myristoylated N-terminal sequence (MANS), corresponding to the first 24 amino acids of MARCKS, which has been shown to inhibit MARCKS function [11,17]. Through utilizing this unique peptide MANS, we have shown that MARCKS plays a critical role in regulating neutrophil function including β2 integrin dependent adhesion, chemotaxis, degranulation and pro-inflammatory cytokine secretion [18-20].

Neutrophil expresses a range of PKC isoforms, including the conventional Ca\(^{2+}\) and diacylglycerol (DAG) – sensitive PKC α and PKC β, the novel Ca\(^{2+}\) - independent PKC δ, and the atypical Ca\(^{2+}\) and DAG – independent PKC ζ [21,21-24]. These PKC isoforms are implicated in various neutrophil function including adhesion [24,25], migration [24,26-28], oxidative burst [29], degranulation [30] and cell apoptosis [31]. However, specific PKC isoforms responsible for MARCKS phosphorylation in neutrophils is still elusive. In the present study, using isolated primary human neutrophils, we report that PKC δ is responsible for phosphorylation of MARCKS in human neutrophils. Utilizing PKC isoform inhibitors Go6976, CG53353, rottlerin and PKC ζ pseudosubstrate for inhibiting PKC α, PKC β, PKC δ and PKC ζ, respectively, we demonstrate that pan-PKC inhibitor, staurosporine and PKC δ inhibitor, rottlerin attenuate MARCKS phosphorylation induced by chemoattractant fMLF.
stimulation in human neutrophils in a dose dependent manner. The IC$_{50}$ of staurosporine and rottlerin for MARCKS phosphorylation were 1.094 μM and 5.709 μM, respectively. Further, subcellular fractionation assay reveals that PKC δ is the only PKC isoform which is activated upon stimulation of human neutrophils with PKC activator PMA or fMLF.

**Materials and Methods**

**Reagents**

Ficoll-Paque Plus and Dextran T500 were from GE Healthcare (Sweden). Dimethyl sulfoxide (Me2SO), f-Met-Leu-Phe (fMLF), phorbol 12-myristate 13-acetate (PMA), Triton-X 100, pepstatin, HEPES, staurosporine, Protein kinase Cζ pseudosubstrate and poly-l-lysine were from Sigma Chemical Co. (St. Louis, MO). Rottlerin, Go6976 and CG53353 were from Calbiochem (Billerica, MA). Powdered phosphate-buffered saline (PBS) and Hank's balanced salt solution (HBSS) were from Life Technologies (Grand Island, NY). Ethylenediamine tetraacetate dihydrate (EDTA) was from Fisher Scientific (Atlanta, GA). Leukotriene B4 (LTB4) was from Cayman Chemical (Ann Arbor, MI). Fetal calf serum (FCS) was obtained from Hyclone (Logan, UT). MARCKS, Phospho-MARCKS, PKC α, PKC δ, PKC ζ primary antibodies and anti-rabbit HRP-conjugated secondary antibody were obtained from Cell Signaling (Beverly, MA). PKC β was from Invitrogen (Frederic, MD). Diisopropylfluorophosphate (DFP) was from BD Biosciences (San Diego, CA).
**Neutrophil Preparation**

Human neutrophils were isolated using a Ficoll gradient centrifugation with dextran-sedimented leukocyte rich plasma from human whole blood. Briefly, approximately 6ml of plasma was layered on 5ml of sterile, endotoxin-free Ficoll-Paque solution and spun at 600g for 20 minutes. Neutrophils were used if they demonstrated greater than 98% viability, as determined by exclusion of trypan blue dye incorporation. Red blood cells were lysed by hypotonic lysis and remaining neutrophils were washed once with HBSS. Cells were re-suspended in HBSS with 20mM HEPES, 8.9 mM sodium bicarbonate, 1mM Ca\(^{2+}\) and 1mM Mg\(^{2+}\) prior to assays (HBSS\(^{++}\)).

**Treatment of PKC Isoform Inhibitors**

Purified human neutrophils (2.5 \(\times\) 10\(^7\) cells/ml) were suspended in HBSS\(^{++}\) and incubated with or without PKC isoform inhibitors for 15 to 30 min with varying concentrations in microcentrifuge tubes and incubated at 37°C. Cells were then stimulated with 100nM fMLF, 50 ng/ml PMA or appropriate vehicle control (VC) for the indicated time periods during incubation at 37°C.

**Preparation of Lysate and Western Blot Assay**

Incubated cells were immediately transferred onto ice and lysed with ice cold RIPA buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM sodium pyrophosphate and 50 mM sodium fluoride] containing protease inhibitors [1mM phenylmethanesulphonylfluoride (PMSF), 100μg/mL pepstatin, 1mM iodoacetamide, and
10μg/mL aprotinin/leupeptin] for 30 mins on ice. After lysis, cell solutions were spun at 13,400 g for 5 mins. Supernatants were collected and protein concentrations were measured using BCA Protein Assay Reagent (Pierce, Rockford, IL). Cell lysate was mixed with 5X sample buffer [25% glycerol, 2% SDS, 60mM Tris-HCl (pH 6.8), 5% β-mercaptoethanol, 0.1% bromophenol blue in diH₂O] and boiled for 5 mins. Equal amount of protein was analyzed in 10% SDS-PAGE. Resolved samples were transferred to Immobilon-P PVDF membrane (Millipore, Billerica, MA) and blocked for 1 hour with 5% non-fat dry milk in tris buffered saline with Tween-20 (TBS/T; 136 μM NaCl, 20 μM Tris-base (pH: 7.6) and 0.1% Tween-20 v/v) prior to overnight incubation with the 1:1000 dilution of phospho MARCKS or MARCKS primary antibody in 5% BSA in TBS/T at 4°C. Membranes were washed in TBS/T and incubated with 1:4000 dilution of anti-rabbit horseradish peroxidase (HRP) secondary antibody (Cell Signaling Technology, Danvers, MA) in 5% non-fat dry milk in TBS/T for 1 hour with gentle agitation. Membranes were washed at room temperature in TBS/T three times for 5 mins and developed using enhanced chemiluminescence and radiograph film. Films were scanned and the density of the bands was measured with densitometric software (Scanalytics, Fairfax, VA).

Subcellular Fractionation

Subcellular fractionation assay was performed as previously described [19]. Briefly, stimulated cells were lysed by sonication in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM DTT). The lysate was centrifuged at 800g for 10 min to remove cell debris and nuclei. Supernatant was then centrifuged at 20,000g for 1 hour. The supernatant was
collected as the cytosolic fraction and the remaining pellet was regarded as the membrane fraction and resuspended in lysis buffer containing 1% Triton X-10. Protein concentration was determined by Protein 660 (Pierce, Rockford, IL) and samples were diluted in 5x sample buffer and boiled for 5 minutes. Equal protein concentrations were loaded onto a 10% SDS-PAGE and western blots were performed for EGFP expression, as described above.

Quantification and Analysis of the Data
Films were scanned and the density of the bands was measured with densitometric software (SigmaScan 5). A student t-test was used to calculate the indicated p values.

Results

MARCKS is phosphorylated by fMLF and PMA in primary human neutrophils
MARCKS has been shown to be an important regulator in human neutrophil migration [19] and its bi-directional movement between the plasma membrane and the cytosol is essential to fulfill its function [14]. Previously, Thelen et al. showed that PKC-dependent phosphorylation is responsible for reversible translocation of MARCKS between the plasma membrane and the cytosol in human neutrophils [12]. Consistent with this finding our results show that the chemoattractant fMLF, a formyl peptide which stimulates PKC-dependent signaling pathways through G protein-coupled receptor (GPCR) [32], induced the rapid and transient phosphorylation of MARCKS (Figure 1A). The phosphorylation occurred 30 seconds after stimulation with 100 nM of fMLF and then decreased, with secondary delayed
phosphorylation peak at 10 mins, which is typically seen in adapting-adaptation responses [33]. Additionally, neutrophils were stimulated with PMA, which bypasses receptor-dependent signals and directly activates PKC by mimicking the second messenger diacylglycerol (DAG) [34]. As illustrated in Figure 1B, 50 ng/ml of PMA resulted in the rapid and persistent phosphorylation of MARCKS. We chose 30 second treatment with 100 nM of fMLF as the optimal stimulation method for the remainder of our studies because fMLF receptor dependent signaling causes a more physiologically relevant phosphorylation and dephosphorylation event.

Inhibition of PKC interferes with MARCKS phosphorylation

We assessed whether the pan PKC inhibitor, staurosporine interferes with MARCKS phosphorylation. Staurosporine attenuates PKC activity by interacting with the catalytic domain of PKC [35]. However, it has been reported that staurosporine has both inhibitory and stimulatory effects on human neutrophils. For example, while some studies have found that staurosporine decreases the chemoattractant-mediated respiratory burst [36,37], others report that it promotes respiratory burst [38,39] and exocytosis in human neutrophils [37]. Purified human neutrophils were pretreated with staurosporine at a range of concentration (50 nM ~ 20 μM) for 30 minutes before cells were stimulated with 100 nM of fMLF. As shown in Figure 2A, pretreatment of staurosporine attenuated fMLF induced MARCKS phosphorylation in human neutrophils in a dose dependent manner. There was a slight, but not significant, increase in phosphorylation at the 100 nM of staurosporine pretreatment (Figure 2B), which supports the observation in a previous study that staurosporine is capable
of enhancing fMLP-stimulated respiratory burst [38]. Indeed, the staurosporine pretreatment significantly reduced phosphorylation of MARCKS with 1 μM or greater as compared with the vehicle control (Figure 2). IC\textsubscript{50} value was determined based on plotting percentage of inhibition, defined as the percentage of reduction in fMLF-induced MARCKS phosphorylation of staurosporine treated cells as compared to vehicle control treated cells, against power of 10 scale of staurosporine concentration in μM. The IC\textsubscript{50} for fMLF induced MARCKS phosphorylation of human neutrophils was 1.094 μM (Figure 2C).

Inhibition of PKC isoform delta (δ) interferes with MARCKS phosphorylation

Neutrophil expresses conventional PKC α and PKC β, the novel PKC δ, and the atypical PKC ζ [40]. We used specific PKC isoform inhibitor Go6976, CG53353, rottlerin and ζ pseudosubstrate for PKC α, PKC β, PKC δ, and PKC ζ, respectively to identify which PKC isoform is responsible for phosphorylating MARCKS in human neutrophils. First, we tested rottlerin which is known as an inhibitor of PKC δ [41].

The inhibitory effect of rottlerin on various PKC isoforms has been reported as follows:
PKC δ (IC\textsubscript{50} = 3~6 μM); PKC α and PKC β (IC\textsubscript{50} = 30~42 μM); PKC ζ (IC\textsubscript{50} = 80~100 μM) [41,42]. Purified human neutrophils were preincubated with rottlerin (1 μM ~ 100 μM) for 15 mins before exposure to 100 nM of fMLF and MARCKS phosphorylation was assessed by western blot. In Figure 3A, pretreatment of rottlerin attenuated fMLF-induced MARCKS phosphorylation in human neutrophils in a dose dependent manner. Rottlerin treatment significantly attenuated phosphorylation of MARCKS with 10 μM or greater as compared with the vehicle control with fMLF stimulation (Figure 3B). The IC\textsubscript{50} of rottlerin for fMLF-
induced MARCKS phosphorylation of human neutrophils was 5.709 μM (Figure 2C). This result indicates that PKC δ is important for fMLF-induced MARCKS phosphorylation in human neutrophils.

Inhibition of PKC isoforms alpha, beta, and zeta dose not interfere with MARCKS phosphorylation

We evaluated whether or not other PKC isoforms play a role in MARCKS phosphorylation in human neutrophils using inhibitors specific for PKC α, PKC β, and PKC ζ. Purified human neutrophils were preincubated with PKC α inhibitor Go6976 (2 ~ 100 μM) for 15 mins [43], PKC β inhibitor CG53353 (also known as DAPH 2) (1 ~ 20 μM) for 30 mins [44,45], and PKC ζ inhibitor PKC ζ pseudosubstrate (1 ~100 μM) for 30 mins [46] before cells were stimulated with fMLF. As illustrated in Figure 4, pretreatment with Go6976, CG53353, and PKC ζ pseudosubstrate had no inhibitory effect on MARCKS phosphorylation induced by 100 nM of fMLF in human neutrophils. These results determine that PKC δ is only PKC isoform which is required for fMLF-induced MARCKS phosphorylation in human neutrophils.

Neutrophil stimulation causes translocation of PKC isoform delta from the cytosol to the plasma membrane

Translocation of PKC isoforms from the cytosol to the membrane compartments when the isoforms are activated is well documented [47] and is a good indicator of intracellular PKC activation [40]. We tested whether stimulation of human neutrophils leads to translocation of
PKC δ isoform or other isoforms (α, β, and ζ) in parallel. Purified human neutrophils were stimulated with 100 nM of fMLF for either 1 second and 2 mins; or 50 ng/ml of PMA for 2 mins. Following stimulation, neutrophils were fractionated into cytosolic and particulated fractions and the relative amount of PKC α, β, δ and ζ isoforms in each fraction were determined by western blot (Figure 5). Fractionation results demonstrated that PKC δ is the only isoform that translocates from the cytosol to the membrane in response to PMA in 2 mins and fMLF in 1 sec. Cells contained the majority of PKC isoforms in the cytosolic fraction, except for PKC β which locates in the membrane fraction. PKC isoforms α, β, and ζ do not appear to translocate from the cytosol to the membrane or vice versa upon stimulation. These data suggest that PKC δ is the only activated PKC isoform when human neutrophils are stimulated by chemoattractant fMLF or the nonphysiologic PKC stimulator PMA.

**Discussion**

Several studies of PKC isoform-specific MARCKS phosphorylation previously performed utilizing cell-free extracts [48,49] indicated conventional PKC α, novel PKC δ and ε, and atypical PKC ζ phosphorylated purified recombinant MARCKS. However, the molecular mechanism of inducing PKC activation *in vitro* may not be the same as those in the intact cell. Moreover, subsequent studies using various intact cells have shown that the major PKC isoform which phosphorylates MARCKS is different depending on the cell type; PKC α, ε and θ in NIH 3T3 fibroblasts [50]; PKC ε in human T84 intestinal epithelia [51]; PKC θ in chick embryonic myoblasts [52]; PKC α in mouse myoblasts [53]; PKC δ in airway
epithelium [54]; PKC δ in rat parotid acinar cells [55]; PKC β in mast cells [56]. However, up to this point, the PKC isoform responsible for MARCKS phosphorylation in intact human neutrophil was unknown.

Thus, MARCKS phosphorylation by various PKC isoforms expressed in human neutrophils was evaluated under physiological condition using specific PKC isoform inhibitors and a subcellular fractionation assay. We demonstrated in this study that PKC δ plays a critical role in MARCKS phosphorylation in human neutrophils. Pan-PKC inhibition using staurosporine and specific isoform PKC δ inhibition using rottlerin significantly attenuates fMLF-induced MARCKS phosphorylation, with an IC₅₀ of 1.094 μM and 5.709 μM, respectively. Furthermore, PKC δ is the only PKC isoform that is activated by stimulation with fMLF and PMA in human neutrophils.

fMLF stimulates PKC-dependent responses through GPCR [32], resulting in rapid phosphorylation of MARCKS and translocation of MARCKS from the plasma membrane to the cytosol [12]. Our MARCKS phosphorylation kinetic assay similarly shows that fMLF treatment in neutrophils caused rapid and transient phosphorylation, maximized at 30 secs, followed by a decrease and then a delayed secondary peak at 10 mins (Figure 1A). Our previous finding demonstrated that fMLF-induced F-actin polymerization in human neutrophils was maximized at 30 secs and returned rapidly to resting levels by 5 mins [19], which is corresponding to fMLF-induced MARCKS phosphorylation kinetics in the present study (Figure 1A). Moreover, MANS peptide treatment of human neutrophils reduced fMLF-induced F-actin polymerization at 30 secs [19]. These results suggest that fMLF-induced
MARCKS phosphorylation may play a critical role in initial neutrophil polarization through regulating cytosolic MARCKS availability and its F-actin crosslinking machinery.

Our original purpose of this study was to identify the PKC isoform(s) precisely involved in MARCKS phosphorylation in human neutrophils. Human neutrophils express the conventional PKC α and PKC β, the novel PKC δ, and the atypical PKC ζ [40]. This classification has been established based on each isoform’s domain structure and regulation; conventional isoforms contain two regulatory domains C1 for binding phosphatidylserine (PS) and diacylglycerol (DAG), and C2 for binding calcium [57]. Novel PKC isoforms lack the C2 domain and are only sensitive to DAG or phorbol ester [58]. Atypical PKCs lack both the C1 and C2, therefore they are not activated by calcium or DAG [58](Ono 1998), but they are activated by PS or phosphoinositide-dependent protein kinase-1 (PDK-1) [59].

Utilizing the pan-PKC inhibitor staurosporine we have shown that inhibiting PKC results in a decrease of MARCKS phosphorylation in a dose dependent manner (Figure 2). Staurosporine has been known as a potent inhibitor of the phospholipid/Ca^{2+} - dependent PKC isoforms with an IC_{50} of 2.7 nM [35]. The differential inhibitory effect of staurosporine (based on the result of the kinase activity of recombinant PKC isoforms) is as follows: PKC α (IC_{50} = 58 nM), β (IC_{50} = 65 nM), γ (IC_{50} = 49 nM), δ (IC_{50} = 325 nM), and ε (IC_{50} = 160 nM) [60]. In our studies with fMLF, staurosporine inhibited MARCKS phosphorylation in a dose-dependent manner with IC_{50} of 1.094 μM, which is approximately 20 times greater than the concentration required to inhibit conventional PKC isoforms. This finding prompted us to consider that Ca^{2+} - insensitive novel PKC δ or atypical PKC ζ might be a potential isoform to phosphorylate MARCKS in human neutrophils. Another interesting finding was that low
dose (50 and 100 nM) fMLF treatment did not affect or slightly increased MARCKS phosphorylation (Figure 2B). This could be related to the previous observations that staurosporine enhances respiratory burst [38,61] and exocytosis [37] in neutrophils under certain condition. Specifically, 100 nM of staurosporine pretreatment maximized respiratory burst in human neutrophils with 100 nM of fMLF stimulation [38], which fits with our finding that MARCKS phosphorylation was maximal with the same conditions (Figure 2B), although the precise mechanism of enhancing fMLF-induced neutrophil activities by staurosporine is unclear. Staurosporine is also known as a potent inhibitor of cAMP- (PKA), cGMP- (PKG) dependent protein kinase (Kᵢ = 7nM) and the protein tyrosine kinase activity (PTK) of p60ᵥ-src (IC₅₀ = 6.4 nM) other than PKC [62]. 100 nM of staurosporine is great enough for inhibiting PKA, PKG or PTK, and not enough for inhibiting novel PKC isoforms. This differential inhibitory effect of staurosporine depending on its concentration range might have affected fMLF-induced neutrophil function. Especially, an inhibitory effect of PKA on neutrophil activation has been well documented by our previous studies [63,64]; therefore enhancement of neutrophil function by inhibiting PKA activities would not be surprising.

When we pretreated neutrophils with various PKC isoform-specific inhibitors, only rottlerin attenuated MARCKS phosphorylation in a dose-dependent manner with IC₅₀ of 5.708 uM (Figure 3 and 4). Rottlerin has been used as a specific PKC δ inhibitor with an IC₅₀ of 3–6 uM, an effect 5~10 times more potent than for conventional PKC isoforms (IC₅₀ of PKC α and β = 30~42 μM) or 30 times more than atypical PKC isoform (IC₅₀ of PKC ζ = 80~100 μM) [41,42]. Although some authors argue that rottlerin is an inappropriate PKC δ inhibitor
others defend its specificity [66-68]. Critics claim that rottlerin uncouples mitochondria, resulting in reduced cellular ATP levels, causing an apparent but non-specific block of PKC δ activity. However, a recent study using dermal fibroblasts has shown that rottlerin-induced upregulation of uncoupling agents is limited. Moreover, the RNA data obtained from PKC δ knock-down or a cell-permeable PKC δ inhibitory peptide corresponds to the microarray results of the inhibition of PKC δ activity by rottlerin [67]. Therefore, our finding that rottlerin, not other PKC isoform inhibitors (Go6976, CG53353, and PKC ζ pseudosubstrate) inhibits fMLF-induced MARCKS phosphorylation suggests that PKC δ is the PKC isoform responsible for inducing MARCKS related neutrophil function.

We further examined PKC δ activity through translocation of PKC isoforms in response to fMLF or PMA (Figure 5). PKC isoform translocation from the cytosol to the membrane has been applied to indicate intracellular PKC activation [40]. In response to PMA and fMLF, only PKC δ moved from the cytosol to the membrane fraction in 2 min and 1 sec, respectively. However, after 2 mins the fMLF-induced PKC δ accumulation in the membrane fraction was no longer present. A previous study demonstrated that PKC isoforms δ and βII in PMA- or fMLF- stimulated human neutrophils were translocated to the membrane containing fraction [40]. This is consistent with our findings, although our results showed transient translocation pattern of PKC δ upon fMLF stimulation and no translocation evidence of PKC β. This slight discrepancy between the two studies could be explained by one or more of the following. 1) They performed the translocation assay in the presence of cytochalasin B. This actin disrupting compound is capable of enhancing the stimulation of fMLF by increasing the intracellular level of DAG [69-72]. This could explain their observation that fMLF-induced
PKC δ accumulation in the membrane fraction as early as 8 secs and the accumulation sustained until 2 mins. Whereas, we did not add cytochalasin B in our PKC isoform translocation experiment, thus our fMLF-induced translocation of PKC δ might have been weak and transient. 2) Experiment buffer conditions were different. We treated the cells with HBSS++ which contains 1mM of Ca²⁺. Previous reports suggest that the intracellular calcium concentration could be increased by the presence of extracellular calcium [73], therefore, Ca²⁺ containing buffer conditions might have caused an accumulation of the PKC β isoform in the membrane fraction prior to stimulation (Figure 5)[74]. Thus, our finding that the translocation of PKC δ from the cytosol to the membrane upon fMLF and PMA stimulation suggests that PKC δ is the only activated PKC isoform which plays a major role in MARCKS phosphorylation in human neutrophils. Further investigation of the involvement of PKC δ isoform in MARCKS phosphorylation would ideally be performed using a molecular genetics approach (including gene-overexpression or knockdown assays); however, isolated primary neutrophils are terminally differentiated and survive less than 48 hours [75] and are therefore limited in their usefulness for genetic manipulation.

In conclusion, the present studies have indicated a major role for PKC δ in phosphorylating MARCKS in human neutrophils. The conclusions of recent studies highlight the importance of MARCKS role in regulating human neutrophil adhesion and migration [19], degranulation [18] and proinflammatory cytokine secretion [20]. Our results add important information to a regulatory pathway that provides potential targets for novel therapeutics designed to target neutrophil mediated inflammatory disorders. Further study to establish the precise mechanism of PKC δ in MARCKS regulation of neutrophil function would be warranted.
Figure 1. Kinetics of MARCKS phosphorylation in response to 100 nM fMLF and 50 ng/ml PMA in human neutrophils. Purified neutrophils were stimulated with (A) 100nM fMLF, (B) 50 ng/ml PMA or (C) vehicle control for the indicated periods of time during a 30 minute incubation at 37 ºC before lysis and western blot analysis for both phospho-MARCKS and total MARCKS. Data are representative of three independent experiments from three separate donors.
Figure 2. MARCKS phosphorylation induced by 100 nM fMLF for 30 secs is effectively inhibited by pan-PKC inhibitor staurosporine in a dose dependent manner in human neutrophils (IC$_{50}$ = 1.094 μM). Purified neutrophils were pre-treated with a range of concentrations of the PKC-inhibitors staurosporine or vehicle control (Me$_2$SO) before stimulation with 100nM fMLF for 30 seconds at 37 °C as described in Materials and Methods. (A) Equal protein amounts were analyzed by Western Blot using anti-phospho MARCKS or total MARCKS antibodies. (B) Densitometry was performed and the density of phospho MARCKS and total MARCKS bands was measured. “% MARCKS phosphorylation” was calculated by dividing the phospho MARCKS band by the total MARCKS and presented as the mean ± SE. Asterisk (*) indicates significant decrease (p<0.05) in phospho MARCKS levels as compared to vehicle control. (C) IC$_{50}$ value was calculated by plotting percentage of MARCKS phosphorylation against power of 10 scale of staurosporine concentration in μM. Data are representative of at least three separate experiments using neutrophils from different donors.
Figure 3. MARCKS phosphorylation induced by 100 nM fMLF for 30 secs is effectively inhibited by PKC δ specific inhibitor, rottlerin in a dose dependent manner in human neutrophils (IC₅₀ = 5.709 μM). Purified neutrophils were pre-treated with a range of concentrations of the PKC δ specific inhibitor rottlerin or vehicle control (Me₂SO) before stimulation with 100nM fMLF for 30 seconds at 37 °C as described in Materials and Methods. (A) Equal protein amounts were analyzed by Western Blot using anti- phospho MARCKS or total MARCKS antibodies. (B) Densitometry was performed and the density of phospho MARCKS and total MARCKS bands was measured. “% MARCKS phosphorylation” was calculated by dividing the phospho MARCKS band by the total MARCKS and presented as the mean ± SE. Asterisk (*) indicates significant decrease (p<0.05) in phospho MARCKS levels as compared to vehicle control. (C) IC₅₀ value was calculated by plotting percentage of MARCKS phosphorylation against power of 10 scale of rottlerin concentration in μM. Data are representative of at least three separate experiments using neutrophils from different donors.
Figure 4. Pretreatment of PKC α, βI inhibitor (Go6976), PKC βI, βII inhibitor (CG53353) or PKC ζ inhibitor (PKC ζ pseudosubstrate) does not affect MARCKS phosphorylation induced by 100 nM fMLF for 30 secs in human neutrophils. Purified neutrophils were pre-treated with a range of concentrations of the PKC α, βI specific inhibitor Go6976 (A), PKC βI, βII inhibitor CG53353 (B), PKC ζ inhibitor PKC ζ pseudosubstrate (C) or vehicle control (Me₂SO) before stimulation with 100nM fMLF for 30 seconds at 37 °C. Equal protein amounts were analyzed by Western Blot using anti- phospho MARCKS or total MARCKS antibodies. Data are representative of at least three separate experiments using neutrophils from different donors.
Figure 5. Subcellular fractionation of human neutrophils. Cells contain majority of PKC α, PKC δ, and PKC ζ in the cytosol and PKC β in the membrane fractions. PKC δ translocates from the cytosol to the membrane in response to stimulation with 50 ng/ml PMA in 2 mins and 100 nM fMLF in 1 sec. Other isoforms do not appear to translocate from the cytosol to the membrane and vice versa. Data are representative of at least three separate experiments using neutrophils from different donors.
REFERENCES


CHAPTER 3

MYRYSTOLAED ALANINE-RICH C-KINASE SUBSTRATE (MARCKS) REGULATES CANINE NEUTROPHIL MIGRATION

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Abstract

To enable recovery from inflammation, neutrophil migration to the site is important to kill bacteria and remove dead cell debris. However, excessive infiltration of neutrophils aggravates inflammation and often leads to devastating results in acute or chronic inflammatory diseases. A more complete understanding of the process of neutrophil migration and adhesion may lead to the identification of targets for novel anti-inflammatory drugs. One such target of interest is Myristoylated Alanine-Rich C Kinase Substrate (MARCKS), which is a major Protein Kinase C (PKC) substrate. We have previously shown that MARCKS is involved in human neutrophil migration and β2-integrin dependent adhesion by utilizing a unique reagent MANS which is the myristoylated N-terminal sequence peptide identical to the first 24 amino acids of MARCKS. We hypothesized that MARCKS regulates canine neutrophil migration and β2-integrin dependent adhesion, and that inhibition of MARCKS (by application of MANS) would inhibit these neutrophil processes. MARCKS was present and rapidly phosphorylated in canine neutrophils upon stimulation by intermediary chemoattractants (LTB4, PAF, and IL8) or end-target chemoattractant C5a, but not fMLF. Either intermediary (LTB4, PAF, and IL8) or end-target (C5a) chemoattractants significantly induced canine neutrophil migration in a dose dependent manner, and MANS pretreatment significantly inhibited this chemotaxis. In addition, MANS treatment significantly reduced β2-integrin dependent adhesion induced by LTB4, PAF, IL8, or C5a. However, control reagent RNS, which is the random N-terminal sequence, had no effect on either chemoattractant-induced migration or β2-integrin dependent adhesion of
canine neutrophils. These results indicate that MARCKS is an important regulator in directed canine neutrophil migration and β2-integrin dependent adhesion.
Introduction

Neutrophils migrate from the blood stream to sites of inflammation in response to signals of bacterial infection or tissue injury from the body. Recovery from these insults requires that neutrophils infiltrate the site of inflammation to clear the exogenous invaders and dead cell debris [1]. Although neutrophils have a positive role in the host defensive systems, a certain degree of host cell damage is inevitable during cell migration, tissue infiltration and bacterial killing [2]. Neutrophil migration into inflamed tissue has been associated with acute and chronic canine inflammatory diseases including atopic dermatitis, chronic obstructive pulmonary disease, hepatic cirrhosis, ischemia-reperfusion injury, and inflammatory bowel disease [3]. Since dogs show species-dependent differences in their neutrophil profile, such as the highest neutrophil/mononuclear cell ratio in the blood and large numbers in the lymphatic system, investigating and characterizing canine neutrophil physiology will provide beneficial information to veterinarians treating canine patients, as well as researchers that utilize canine models of disease [4,5].

Myristoylated alanine-rich C kinase substrate (MARCKS) is a protein kinase C (PKC) substrate that is ubiquitously expressed and involved in various functions such as actin filament crosslinking, mucin secretion, neural development and cell migration [6-10]. In most cell types, MARCKS is associated with the cell membrane in the resting state and is translocated into the cytosol when it is phosphorylated by PKC or associated with calmodulin (CaM)[9]. MARCKS is associated with the plasma membrane by 2 important binding forces: insertion of the myristoylated N-terminus moiety and electrostatic interactions between the
positively charged effector domain (ED) (or phosphorylation site domain (PSD)) and negatively charged phospholipids of plasma membrane [9]. While tethered on the plasma membrane, MARCKS sequesters phosphatidylinositol 4,5-bisphosphate (PIP2), a minor lipid of the plasma membrane that controls the activity of numerous proteins and serves as a source of second messengers [11]. MARCKS-mediated PIP2 sequestration interferes with PIP2 hydrolysis by protein lipase C (PLC) isotypes, thereby blocking PIP2 dependent signaling pathways [12,13]. Translocation of MARCKS into the cytosol releases PIP2 masking, which allows actin-binding proteins and/or cell polymerizing factors to access to the plasma membrane. This event results in changes in cell morphology and initiation of cell migration [14-16]. Previously, through use of a known MARCKS inhibitor developed in our lab, the MANS peptide, we have shown a role for MARCKS in human neutrophil adhesion and migration. Migration and β2-integrin dependent adhesion of MANS-treated human neutrophils was significantly decreased in the presence of end stage (fMLF) or intermediate stage (IL-8 and LTB4) chemoattractants [17]. We postulated that the MARCKS protein was also essential to canine neutrophil migration and β2-integrin dependent adhesion and tested this hypothesis utilizing the previously developed MANS peptide. Our results show that MANS significantly decreased canine neutrophil migration and adhesion towards well known chemoattractants LTB4, PAF, IL8, and C5a, and that MARCKS inhibition is a viable target for future anti-inflammatory drug development.
Materials and Methods

Reagents

Calcein was from Anaspec (Fremont, CA). Platelet-activating factor (PAF) was from Cayman Chemical (Ann Arbor, MI), Leukotriene B4 (LTB4), N-formyl-methionyl-leucyl-phenylalanine (fMLF), interleukin 8 (IL8), ethylenediamine tetraacetate dehydrate (EDTA), ethylene glycol tetraacetic acid (EGTA), and albumin solution from bovine serum (BSA) were from Sigma (St. Louis, MO). Phorbol 12-myristate 13-acetate (PMA) was from ACROS organics (Geel, Belgium). C5a was from R&D systems (Minneapolis, MN). cOmplete Mini, ULTRA, and PhosSTOP Tablets were from Roche (Mannheim, Germany). Pierce 660 nm Protein Assay Reagent and SuperSignal West Pico Chemiluminescent Substrate were from Thermo Scientific (Rockford, IL). MARCKS and phosphoMARCKS Abs were from Cell Signaling (Danvers, VA). Ammonium chloride (NH4Cl), deoxycholic acid, and tris base were from Fisher Scientific (Fair Lawn, NJ). Ficoll-PaqueTM PLUS was from GE Healthcare (Sweden). Fetal bovine serum (FBS) was from Gemini bio-product (West Sacramento, CA). Dulbecco’s phosphate-buffered saline (PBS) was from GIBCO (Gland Island, NY). Non-fat dry milk was from Labscientific (Livingston, NJ). Hanks’ balanced salt solution (HBSS) was from Mediatech, Inc. (Manassas, VA).

Peptides

Synthesis of both the myristoylated N-terminal sequence (MANS) which consists of sequence identical to the first 24 amino acids of MARCKS : myristic acid –
GAQFSKTAAKGEAAAERPGEAAVA, and the random N-terminal sequence (RNS) peptides (myristic acid – GTAPAAEGAGAEVKRASAEAKQAF) was performed at Genemed Synthesis, Inc. (San Francisco, CA) as previously described [8].

**Canine Neutrophil Preparation**

Peripheral blood was collected from healthy dogs who were not currently on any medication via jugular venipuncture into vacutainer ™ tubes containing ACD (Becton-Dickinson. Franklin Lakes, NJ). Whole blood was diluted 1:1 with sterile PBS, overlayed with endotoxin-tested Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) in sterile 50 ml conical tube, and centrifuged at 600g for 20 mins. Neutrophils were harvested from the bottom of the tube. Red blood cells were lysed with ACK lysis buffer (150 mM NH4Cl, 10 mM KHCO3, and 0.1 mM Na2EDTA) and cells were washed twice in HBSS. Neutrophils were used if they demonstrated greater than 98% viability, as determined by exclusion of trypan blue dye incorporation.

**Preparation of Lysate**

Neutrophils (2.5 × 10⁷/ml) were resuspended in HBSS++. Cells were stimulated with 10 nM LTB4, 100 nM PAF, 100 nM fMLF, 10 nM IL8, 100 nM C5a, 50 ng/ml PMA or vehicle control (EtOH or H2O) for 30 secs at 37 °C. After stimulation, samples were immediately placed on ice. Samples were microcentrifuged for 10 mins at 6,000 g and the pellet was washed one time with ice cold PBS. Cells were resuspended in RIPA buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium
deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4) containing protease inhibitor cocktail cOmplete Mini (Roche), 5 mM diisopropylfluorophosphate and phosphatase inhibitor cocktail PhoSTOP (Roche) and placed on ice for 30 min with agitation. Samples were clarified by microcentrifugation for 5 min at 13,000 g. Total protein in the lysates was determined by Protein Assay ReagentTM (Thermo Scientific). Sample aliquots were stored at -20°C until SDS-PAGE analysis.

**Western Blot Assay**

Equal protein concentrations were analyzed by SDS-PAGE. Resolved samples were transferred to Immobilon-P PVDF transfer membrane (Millipore, Billerica, MA) and blocked for 1 h with 5% nonfat dry milk before overnight incubation with the indicated primary Ab in TBS/T (137 mM NaCl, 20 mM Tris-HCl (pH 7.6), 0.1% Tween-20) with 5% BSA. Membranes were washed a minimum of three times for 5 min each with TBS/T. Membranes were then incubated with HRP-conjugated secondary Ab in 5% NFDM for 2 h at room temperature and washed a minimum of three times with TBS/T for 7 min each. Immunoreactive proteins were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) following manufacturers protocol. Exposed radiographic films were developed, and electronically scanned. Data are presented from representative experiments (immunoblots).
Migration Assay

Neutrophils labeled with the cell-permeant fluorescent dye calcein (2 µg/ml) for 30 minutes at RT were resuspended in HBSS++ with 2% FBS to a final concentration of $2.0 \times 10^6$ /ml. Cells were pretreated with 50 mM of peptide or PBS for 30 minutes at 37°C. A total of $4.0 \times 10^4$ cells were placed on a 5-µm pore size membrane of a ChemoTx® plate (Neuro Probe, Inc., Gaithersburg, MD). Lower wells of the plate were filled with HBSS with or without chemoattractant. $4.0 \times 10^4$ cells were loaded in standard wells which were used as 100% migration control. Cells were allowed to migrate for 1 hour at 37°C. After incubation, cells on the top of the filter were washed away with PBS and 0.5 M EDTA added to the top of the filter for 10 minutes to detach adherent cells. The plate was then centrifuged at 170 g for 1 minute, the filter removed, and fluorescence measured in the lower wells (485 nm excitation, 530 nm emission wavelengths) using an fMax fluorescence plate reader (Molecular Devices, Sunnyvale, CA). Percent migration was determined by dividing the fluorescence of each well by the fluorescence of the standard wells containing $4.0 \times 10^4$ cells.

Adhesion Assay

Neutrophils (1.0 × 10^7/ml) were suspended in HBSS and incubated with calcein (2 µg/ml) for 30 minutes at RT. Cells were washed once and resuspended in HBSS++ with 2% FBS to a final concentration of $2.0 \times 10^6$ /ml. Cells were pretreated with 50 mM of peptide (MANS or RNS) or PBS for 30 minutes at 37°C. $1.4 \times 10^5$ Cells were then added in triplicate to Immulon 2 plates (Dynatech, Chantilly, VA) coated with 5% FCS in PBS, as previously described. After incubation at 37°C for 10 minutes, adhesion was stimulated with 10 nM
LTB4, 100 nM PAF, 100 nM IL8 or 100 nM C5a for 3 minutes at 37°C. Fluorescence was measured (485 nm excitation, 530 nm emission wavelengths) using an fMax fluorescence plate reader (Molecular Devices) before and after serial washes with 70 µl PBS to dislodge nonadherent cells. Fluorescence after washing was divided by initial fluorescence to calculate percent adhesion.

**Statistical Analysis**

Data were reported as mean ± SEM. Data were analyzed by Student’s *t* test assuming equal variance, or one-way ANOVA followed by Holm-Sidak method using SigmaPlot® 12 software (Systat software, San Jose, CA). The level of significance used was *p* < 0.05.

**Results**

*Chemoattactants activate MARCKS in canine neutrophil*

We previously demonstrated that MARCKS is an important regulator in human neutrophil migration [17]. MARCKS not only functions to sequester PIP2, an important second messenger in cell signaling, but it also binds to actin filaments, resulting in modulation of cell morphology and migration [9]. Phosphorylation of MARCKS is required for the translocation of MARCKS from the plasma membrane to the cytosol, while dephosphorylation returns MARCKS from the cytosol to the plasma membrane. The cycling of MARCKS subcellular localization is necessary for exerting MARCKS function within the
cell [7]. To verify that chemoattractants induce MARCKS phosphorylation in canine neutrophils, we stimulated purified canine neutrophils with LTB4, PAF, fMLF, IL8 and C5a; which are known as potent chemoattractants for human neutrophils. Total MARCKS and phospho MARCKS protein levels were screened with Western blot analysis as described in Section 2.1. Stimulation of the cells with LTB4, PAF, IL8 and C5a led to strong MARCKS phosphorylation compared to vehicle controls, while fMLF did not affect the level of MARCKS phosphorylation (Fig. 1). PMA stimulation was used as a positive MARCKS phosphorylation control. Total MARCKS levels remained unchanged regardless of cell stimulation.

**Chemoattractants induce canine neutrophil migration**

The ChemoTx® plate (Neuro Probe, Inc., Gaithersburg, MD) was used to determine chemotaxis of canine neutrophils towards independent chemoattractants. Dose-dependent relationships for induction of canine neutrophil chemotaxis were obtained for LTB4, PAF, IL8 and C5a (Fig. 2A and B). All four chemoattractants that induced MARCKS phosphorylation in Section 3.1 caused directed cell migration in a dose dependent manner. LTB4 concentrations from 0.01 to 1000 nM caused significant neutrophil chemotaxis, with maximal percent migration observed at 10 nM LTB4 (Fig. 2A). PAF, IL8 and C5a showed induction of directed migration in a dose dependent manner with maximal percent migration observed at 100 nM, 10 nM and 100 nM, respectively (Fig. 2B). There was no effect of fMLF at any concentration on canine neutrophil migration. This finding is consistent with previous reports that fMLF has no effect on canine neutrophils [18,19]. HBSS only or vehicle
(VC) control treated cells consistently showed background/random migration of about 20%, similar to the level of fMLF treated cells.

**MARCKS inhibition decreases canine neutrophil migration**

To characterize MARCKS function in canine neutrophil migration, we applied MANS peptide, a known MARCKS inhibitor, to ChemoTx® plate based migration assays [17]. Pretreatment of isolated neutrophils with varying concentrations of MANS peptide significantly decreased directed canine neutrophil migration towards chemoattractants LTB4 or IL8 in a concentration dependent manner, while the control peptide RNS had no effect. Less than 12% of cells randomly migrated into the lower wells without chemoattractants in either MANS or RNS pretreatment groups. Increasing inhibition of chemotaxis by the MANS peptide was observed at concentrations from 10 - 50 µM in both LTB4 and IL8 stimulated neutrophils. At 50 uM MANS pretreatment the percentage of migrated cells in the LTB4 and IL8 stimulated groups was no different than the percent migration seen in unstimulated cells (Fig. 3A).

Using the optimized chemoattractant concentrations determined in Section 3.2, we next evaluated the effect of MARCKS inhibition, through pretreatment with MANS peptide, on canine neutrophil chemotaxis toward various chemoattractants. Chemotaxis of peptide and VC treated canine neutrophils induced by LTB4 (10 nM), PAF (100 nM), IL8 (10 nM) and C5a (100 nM) was evaluated (Fig. 3B). A concentration of 50 uM MANS was chosen for maximal inhibition of chemotaxis (see Section 3.2). Canine neutrophils treated with the control peptide RNS or PBS showed about 50% migration towards PAF and 70-80%
migration towards LTB4, IL8 and C5a; while the 50 µM MANS treatment group had less than 20% of the cells that migrated in the chemoattractant containing wells.

**MARCKS inhibition significantly reduces chemoattractant-induced Adhesion**

We previously reported that MANS treatment significantly attenuates adhesion of fMLF- or PMA- activated human neutrophils on 5% FBS coated plates, implicating MARCKS as an important regulator of β2 integrin dependent adhesion in human neutrophils [17,20,21]. To evaluate the inhibitory effect of MANS on β2 integrin dependent adhesion in canine neutrophils, we utilized an Immulon® 2 Plate based adhesion assay. LTB4, PAF, IL8 and C5a induced similar levels of percent adhesion in PBS and RNS treated canine neutrophils (15%, 12%, 12%, and 18% respectively with PBS pretreatment vs. 15%, 15%, 9%, and 15%, respectively with RNS pretreatment). MANS pretreatment significantly reduced all chemoattractant-induced adhesion to less than 2% (Fig. 4). Unstimulated (HBSS) canine neutrophils which were pretreated with either PBS or RNS showed approximately 6% background adhesion, which also dropped to less than 2% with MANS treatment.

**Discussion**

The neutrophil constitutes the first line of defense against infectious agents and its migration to the site of infection is vital for host survival [22]. However, the neutrophil has a variety of toxic weapons including hydrolytic, oxidative, and pore-forming molecules which are able to cause serious collateral host tissue destruction [2]. Furthermore, overwhelming neutrophil
recruitment is a significant component of severity in many diseases such as atopic dermatitis, ischemic injury/infarction, trauma, autoimmunity, liver injury and obstructive pulmonary disorders [1,23-27]. Therefore, we strive to understand the mechanisms of neutrophil migration in response to inflammation as a first step toward developing new neutrophil targeting anti-inflammatory therapies. Because MARCKS has been shown to regulate migration, adhesion and degranulation in human neutrophils [17,28], we have used a known MARCKS inhibitor, the MANS peptide, to investigate the role of MARCKS in canine neutrophil migration and adhesion.

In the present study, we have shown a role for MARCKS in canine neutrophil migration and adhesion. Pretreatment of canine neutrophils with MANS, a synthetic, myristolated, cell permeant peptide identical to the first 24 amino acids of MARCKS, significantly inhibited migration and adhesion of primary canine neutrophils induced by various chemoattractants. In this study, we utilized intermediary chemoattractants such as LTB4, PAF and IL8 (which activate the cell through PI3K/Akt signaling pathway) and end target chemoattractants fMLF and C5a (which are p38 MAPK dependent). All chemoattractants caused significant migration and adhesion except for fMLF, which is known to not stimulate the canine neutrophil [18,19]. MARCKS inhibition significantly blocked directed canine neutrophil migration towards both end target (C5a) and intermediary (LTB4, PAF and IL8) chemoattractants in a dose dependent manner. In addition, MANS pretreatment abrogated β2-integrin dependent adhesion in chemoattractant-stimulated canine neutrophils. These findings correspond to results that we previously reported in human neutrophils [17]. A new possibility emerging from this study is that MANS and compounds derived from MANS
could be useful in dogs to treat serious inflammatory diseases or other neutrophil related disorders. In addition, this study provides further evidence for parallels between canine and human inflammatory cell behavior, which is important for researchers interested in developing human disease models.

Our results showed LTB4, PAF, IL8 and C5a increased percent canine neutrophil migration in a dose dependent manner. The effect of the chemoattractants used in the present study has been previously well characterized [18,19,29]. Research using human neutrophils has shown that chemoattractant signals are prioritized through distinct pathways. End target chemoattractants including bacterial products (fMLF) and complement fragments (C5a) signal through p38 MAPK and are dominant over host-derived intermediary chemoattractants (LTB4, PAF and IL8) which rely on PI3K/Akt signaling [30-33]. In order for neutrophils to migrate actively from blood circulation to the site of inflammation, they must have firm adhesion to the vascular endothelial cells via adhesion molecules called β2-integrins. Subsequent transmigration and chemotaxis also require these integrins as well. Two important β2-integrins are considered in these steps: LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) [34]. Mac-1 has been shown to be predominant on the surface of neutrophils [35] and to increase in surface expression in response to inflammatory stimuli [36] through secretory vesicle transport [37]; but this surface up-regulation does not seem to be essential for Mac-1 adhesion during chemotaxis [38]. However, the use of blocking antibodies that target the high affinity conformation of Mac-1 resulted in almost complete blockade of human neutrophil adhesion to various substrates [39]. This result suggests that the increase in integrin affinity is far more critical than the density in regulating neutrophil adhesion and
migration [40]. Although the predominant integrin expressed on neutrophils is Mac-1, LFA-1 has been reported to work superior to Mac-1 in mediating the firm adhesion of neutrophils [41]. Several studies indicate that Mac-1 and LFA-1 are distinctly regulated; end target stimulants such as fMLF and C5a mediate Mac-1 dependent migration through p38 MAPK pathway, whereas LFA-1 is regulated via PI3K/Akt upon stimulation by intermediary chemoattractant IL8 and LTB4 [42-44].

The present findings are also in accord with a previous study on canine neutrophils that Formyl-Met(M)-Leu(L)-Phe(F) (fMLF) has no effect in the dog [19]. fMLF is widely used as a representative bacteria derived [45] and an end-target chemoattractant [43], but the lack of response on neutrophil migration has been reported in some species including pigs [46], cattle [47], cats [48], and horses [49]. Due to the lack of fMLF receptor on canine neutrophil, migration towards end-target bacteria is mostly C5a dependent in the dog [29].

MARCKS has been reported to play a substantial role in regulating mucin secretion in bronchial epithelial cells, leukocyte degranulation, adhesion, and migration through inhibition by MANS, a synthetic peptide identical to the first 24 N-terminus sequences of MARCKS [8,17,28,50,51]. MANS has been shown to affect MARCKS function in several ways. First, MANS treatment disrupted the association of MARCKS with membranes of mucin granules in the goblet cells in a mouse model of airway inflammation. Second, pretreatment of human bronchial epithelial cells with MANS interfered with MARKCS localization in mucin granule membranes. Finally, MANS treatment of unstimulated primary human neutrophils isolated from peripheral blood dislocated MARCKS from plasma membrane to the cytosol. These lines of evidence suggest that MANS inhibits MARCKS
activity in the cell by interfering with MARCKS association with the membrane. While the myristoyl moiety of MARCKS is absolutely important for associating with membrane compartment [52], myristoylated proteins including MARCKS are also believed to target specific receptors at the cytosolic face of the plasma membrane [53-56]. Considering MANS shares a peptide sequences identical to the N-terminus of MARCKS, it is reasonable to hypothesize that MANS is inhibiting MARCKS function specifically by competing with MARCKS for its specific receptor.

We previously demonstrated that MANS treatment reduced directed chemotaxis and β2 integrin dependent adhesion of human neutrophils [17], which is consistent with our present findings that MANS pretreatment significantly inhibited canine neutrophil migration and β2 integrin dependent adhesion upon stimulation with both intermediary and end-target chemoattractants. These results indicate that MARCKS inhibition via MANS treatment blocks both adhesion and migration; processes that utilize PI3K/Akt and p38 MAPK pathways. Interestingly, our previous findings and other unpublished data have shown that MANS treatment does not affect many other cell functions. For example, MANS pretreatment did not affect chemoattractant or PMA-induced β2-integrin surface upregulation, actin polarization, cell spreading, MARCKS phosphorylation, or AKT phosphorylation in neutrophils, which are all processes associated with neutrophil chemotaxis [17]. One essential mechanism of neutrophil adhesion and migration in which MARCKS role has not yet been investigated is β2 integrin activation.

When cells are stimulated by chemoattractants, integrins undergo a conformational change defined as “integrin activation” [57], which increases their affinity for ligands and enhances
their activity [58]. As stated above, an increase in binding affinity of integrins is far more important for substrate binding and subsequent migration than their density. Integrin activation is controlled by the binding of the protein talin to the integrin β-subunit [59]. This process is mediated by the membrane phospholipid PIP2 [60]. Since MARCKS is able to regulate local PIP2 availability by sequestering PIP2 through its polylysine region [11], it is likely that MARCKS plays a role in the talin mediated activation of β2-integrins. Additionally, MARCKS is known as an important factor in stabilizing focal adhesions in fibroblasts and myoblasts [61,62] and dynamic adhesions in highly motile melanoma cells [63]. MANS may disrupt recruitment of factors required for stabilizing focal adhesion by mimicking MARCKS, resulting in decreased migration and adhesion. Further research is required to determine the mechanism by which MARCKS (and MANS) regulate neutrophil migration and adhesion.

The dog is one of the most commonly treated species in the field of veterinary medicine, as well as a frequently used translational model for research. [64-67]. The dog’s unique blood profile includes the the highest neutrophil/mononuclear cell ratio in the blood and large numbers of neutrophils in the lymphatic system [18]. In addition, it has been shown that canine neutrophils spontaneously release a factor which suppresses proliferation of lymphocyte [4]. These species specific characteristics seem to indicate a dominant role of neutrophils in inflammatory reactions in the dog. Therefore, therapeutics aimed at interfering with neutrophil migration and adhesion may be of significant benefit in treating numerous canine diseases. In summary, the results presented here suggest that MARCKS is an important regulator in canine neutrophil migration and β2 integrin adhesion. Further
investigation into the MANS peptide, or compounds derived from MANS could yield promising therapeutic agents for treating serious inflammatory diseases in the dog.
Figure 1. Chemoattractants LTB4, PAF, IL8 and C5a, but not fMLF activate MARCKS in canine neutrophils. Purified neutrophils from healthy dog were stimulated with 10 nM LTB4, 100 nM PAF, 100 nM fMLF, 10 nM IL8, 100 nM C5a, 50 ng/ml PMA or vehicle control at 37 °C for 30 sec. Lysate preparation and western blot were performed as described in Section 2.4 and 2.5. Data are representative of three independent experiments from three separate donors.
Figure 2. Chemoattractants induce directed-canine neutrophil migration in a dose dependent manner. The effect of varying concentration of (A) LTB4 (0.001 nM to 1000 nM), and (B) PAF, fMLF, IL8 and C5a (0.1 nM to 100 nM) on canine neutrophil migration were shown. Purified calcein-labeled canine neutrophils were allowed to migrate for 1 hour (37 ºC, 5% CO₂) towards the chemoattractant as described in Section 2.6. Percent migration was calculated by dividing the fluorescence of each well by the fluorescence of standard wells. Values are expressed as the mean ± SEM. Significant increase (*p < 0.05, **P < 0.001) in migration compared to vehicle control. Data are representative of three independent experiments from three separate donors.
Figure 3. MANS inhibits chemoattractant-induced migration. (A) Purified calcein-labeled canine neutrophils were pretreated with MANS or RNS in the concentration range 10 μM to 50 μM for 30 min then allowed to migrate for 1 hour (37 ºC, 5% CO₂) towards vehicle (HBSS), 10 nM LTB4 or 10 nM IL8. There was a peptide concentration-dependent decrease in LTB4 or IL8-induced percent migration after treatment with concentration of MANS greater than 10 μM, but no decrease with RNS treatment. Asterisks or pound sign indicate a significant different (*,#p < 0.05, **,#P < 0.001) in migration of MANS-treated cells stimulated with either LTB4(*) or IL8(#) compared to RNS at the same concentration of peptide. Data are representative of four independent experiments from four separate donors. (B) Purified calcein-labeled canine neutrophils were pretreated with 50 μM of MANS, 50 μM of RNS or PBS for 30 min then allowed to migrate for 1 hour (37 ºC, 5% CO₂) towards 10 nM LTB4, 100 nM PAF, 100 nM fMLF, 10nM IL8, 100 nM C5a, and vehicle control. Asterisks indicate a significant difference (*p < 0.05, **P < 0.001) between percent migration of cells treated with MANS and RNS. Values are expressed as mean ± SEM of the percentage of the input cells migrating to the lower chamber. Data are representative of three independent experiments using separate donors.
Figure 4. MANS reduces β2-integrin dependent adhesion. Purified calcein-labeled canine neutrophils were pretreated with 50 μM of MANS, 50 μM of RNS or PBS at 37 °C for 30 min then plated in FBS-coated wells and allowed to adhere at 37 °C for 10 min. Cells were then stimulated with 10 nM LTB4, 100 nM PAF, 10 nM IL8, 100 nM C5a, or vehicle control at 37 °C for 3 min. Percent adhesion was calculated by dividing the fluorescence of each well after washing by the fluorescence of each wells before washing. Values are the mean ± SEM of percentage adhesion. Asterisks indicate a significant difference (*p < 0.05) in percent adhesion of cells treated with MANS compared with RNS. Data are representative of three independent experiments from three separate donors.
REFERENCES


CHAPTER 4

FIBROBLAST MIGRATION IS REGULATED BY MYRISTOYLATED ALANINE-RICH C-KINASE SUBSTRATE (MARCKS) PROTEIN

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(Authors’ contributions: scratch wounding assay, transmembrane chemotaxis assay, PDGF stimulation assay, CSFE proliferation assay using peptides were performed by Dr. Laura Ott; live-cell fluorescence microscopy and image analysis were performed by Adam Melvin; genetic structure-function analysis including subcellular fractionation assay, scratch wounding assay, and transmembrane chemotaxis assay using recombinant plasmids and siRNA were performed by Eui Jae Sung)

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Abstract

Myristoylated alanine-rich C-kinase substrate (MARCKS) is a ubiquitously expressed substrate of protein kinase C (PKC) that is involved in reorganization of the actin cytoskeleton. We hypothesized that MARCKS is involved in regulation of fibroblast migration and addressed this hypothesis by utilizing a unique reagent developed in this laboratory, the MANS peptide. The MANS peptide is a myristoylated cell permeable peptide corresponding to the first 24-amino acids of MARCKS that inhibits MARCKS function. Treatment of NIH-3T3 fibroblasts with the MANS peptide attenuated cell migration in scratch wounding assays, while a myristoylated, missense control peptide (RNS) had no effect. Neither MANS nor RNS peptide treatment altered NIH-3T3 cell proliferation within the parameters of the scratch assay. MANS peptide treatment also resulted in inhibited NIH-3T3 chemotaxis towards the chemoattractant platelet-derived growth factor-BB (PDGF-BB), with no effect observed with RNS treatment. Interestingly, live cell imaging of PDGF-BB induced chemotaxis demonstrated that MANS peptide treatment resulted in weak chemotactic fidelity compared to RNS treated cells. MANS and RNS peptides did not affect PDGF-BB induced phosphorylation of MARCKS or phosphoinositide 3-kinase (PI3K) signaling, as measured by Akt phosphorylation. Further, no difference in cell migration was observed in NIH-3T3 fibroblasts that were transfected with MARCKS siRNAs with or without MANS peptide treatment. Genetic structure-function analysis revealed that MANS peptide-mediated attenuation of NIH-3T3 cell migration does not require the presence of the myristic acid moiety on the amino-terminus. Expression of either MANS or unmyristoylated
MANS (UMANS) C-terminal EGFP fusion proteins resulted in similar levels of attenuated cell migration as observed with MANS peptide treatment. Taken together, these data demonstrate that MARCKS regulates cell migration and suggests that MARCKS-mediated regulation of fibroblast migration involves the MARCKS amino-terminus. Further, this data confirms that the MANS peptide specifically targets MARCKS and demonstrates that MANS mediated inhibition of cell migration occurs independent of myristoylation.
Introduction

Myristoylated alanine-rich C-kinase substrate (MARCKS) is a ubiquitously expressed protein kinase C (PKC) substrate that binds both actin and calmodulin (CaM) and regulates actin dynamics. MARCKS is cooperatively tethered to cell membranes by insertion of its myristoylated amino-terminus as well as electrostatic interactions between the basic effector domain of MARCKS and acidic phospholipids of the plasma membrane [1,2]. Phosphorylation of MARCKS by PKC, or CaM binding, results in the release of MARCKS from the plasma membrane into the cytosol in a process called the “myristoyl-electrostatic switch” mechanism [3]. Dephosphorylation or release of CaM results in the ability of MARCKS to return to the plasma membrane. This membrane to cytosol shuttling, or bi-lateral translocation of MARCKS, has been associated with the reorganization of the actin cytoskeleton [4,5], with various cellular processes regulated by MARCKS, including: endo-[6], exo- [7], and phagocytosis [8,9], as well as cell migration [10,11].

MARCKS is involved in regulation of motility in various cell types including fibroblasts [12], myoblasts [13], human embryonic kidney cells [14], human hepatic stellate cells[10], vascular smooth muscle cells [15], neutrophils [16], mesenchymal stem cells [17] and various cancer cells [11,18,19]. One of the initial steps during cell migration is adherence of cells to the extracellular matrix, and a role for MARCKS in regulating such cell adhesion has been established. Expression of a mutated MARCKS in which the myristoyl-electrostatic switch mechanism is altered (thus inhibiting MARCKS bi-lateral translocation) resulted in abrogated cell adhesion and spreading [12,13]. Glioblastoma multiforme cells
that express a constitutively active variant of the epidermal growth factor receptor (EGFR) underwent decreased adhesion, spreading and invasion when transfected with a siRNA targeting MARCKS [19]. Additionally, MARCKS is localized to focal adhesions during α5 integrin myoblast attachment and spreading and silencing of MARCKS resulted in decreased myoblast spreading [20].

Recently, a unique reagent called MANS, a myristoylated cell permeant peptide corresponding to the first 24-amino acids of MARCKS, has been used to demonstrate a role for MARCKS, specifically its myristoylated amino-terminus, in regulating the migration of neutrophils [16] and mesenchymal stem cells [17]. These results raised the question as to which aspect(s) of the MANS peptide, as well as the amino-terminus of MARCKS, could be involved in regulation of cell migration, with particular interest in amino-terminal myristoylation, given its role in membrane attachment [21,22]. Fibroblasts, as opposed to neutrophils as previously described [16], were utilized in these experiments for two reasons. First, to determine if myristoylation of MANS is involved in regulating cell migration, a genetic structure-function analysis was performed. Fibroblasts are more suitable for these studies as they are a migratory cell type that are easily transfected, unlike terminally differentiated and difficult to transfect neutrophils. Second, fibroblasts were used in these experiments because they solely express MARCKS [25,34] while neutrophils, a phagocytic leukocyte similar to macrophages, may express MARCKS-like protein (MLP; also called MARCKS-related protein (MRP) or MacMARCKS) in addition to MARCKS [23-25]. Both MARCKS and MLP have a myristoylated amino-terminus with approximately 50% homology [21] and are involved in the regulation of cell migration [12,13,26]. Thus, our
previous work demonstrating that MANS peptide treatment inhibits neutrophil migration [16] does not rule out the possible involvement of MLP.

Herein, the MANS peptide was utilized to demonstrate that MARCKS is integrally involved in the regulation of directed fibroblast migration, as measured by scratch wounding and PDGF-BB–induced chemotaxis assays. Further, siRNAs that target MARCKS were used to demonstrate that MARCKS expression is not essential to cell migration and that the MANS peptide specifically inhibits MARCKS function. Additional genetic structure-function analysis revealed that MANS peptide mediated inhibition of NIH-3T3 cell migration does not require the presence of the myristic acid moiety on the amino-terminus, as expression of either MANS or unmyristoylated MANS (UMANS) C-terminal EGFP fusion proteins resulted in similar levels of attenuated migration. Taken together, the results of these studies support our previous findings that MARCKS regulates cell migration although MARCKS expression is not essential to the process of cell migration. These studies also demonstrate that the MANS peptide specifically targets MARCKS and that myristoylation of the MANS peptide is not required for MANS peptide-mediated inhibition of fibroblast migration.
Results

*MANS peptide attenuates migration of fibroblasts on fibronectin or collagen substrates*

As illustrated in Figure 1, MANS peptide treatment significantly decreased NIH-3T3 fibroblast migration on either fibronectin (Figures 1A & B) or collagen (Figure 1C) substrates in a concentration-dependent manner. Treatment of the cells with the myristoylated missense scrambled control (RNS) peptide did not alter the ability of fibroblasts to migrate on either fibronectin or collagen (Figures 1B & C). As a positive control for inhibition of migration, fibroblasts were incubated with the phosphoinositide 3-kinase (PI3K) inhibitor wortmannin, which has been shown previously to inhibit NIH-3T3 fibroblast migration [27]. As expected, wortmannin treatment resulted in significantly decreased fibroblast migration compared to non-treated or RNS treated cells, while wortmannin and MANS treated cells had a similar level of inhibition (Figure 1). Taken together, these results are the first to demonstrate that the MANS peptide inhibits fibroblast migration. These results also suggest that the amino-terminus of MARCKS may be involved in regulating NIH-3T3 fibroblast migration, as we have previously described in neutrophils [16] and mesenchymal stem cells [17]. Based on these results, additional studies utilized a concentration of 50 μM MANS or RNS.

To rule out that the results observed in our scratch-wounding assay were not due to altered cell proliferation, we performed carboxyfluorescein succinimidyl ester (CFSE) proliferation assays. Briefly, we incubated CFSE-labeled fibroblasts in fibronectin coated plates for 18 hours (duration of scratch assay) in the presence or absence of 50 μM MANS,
50 μM RNS, vehicle control (VC; PBS) or wortmannin. As shown in figure 2, no difference was observed in NIH-3T3 proliferation in MANS or RNS treated cells compared to non-treated, wortmannin or VC treated cells. Thus, these results confirm that MANS peptide treatment does not alter cell proliferation and that the results observed in figure 1 are due to altered cell migration and not proliferation.

**MANS inhibits PDGF-BB stimulated fibroblast chemotaxis**

Fibroblasts migrate by both cell-contact cues, as demonstrated in the wound healing process, as well as by directional chemotaxis towards chemoattractants such as PDGF-BB [28-30]. In the past, PI3K has been thought to be the main mediator of cell migration, and several reports have established that PI3K is involved in regulating PDGF-BB mediated fibroblast migration [31-33]. However, PDGF-BB stimulation of Swiss 3T3 fibroblasts and human hepatic stellate cells also results in phosphorylation and membrane to cytosol translocation of MARCKS [10,34-36], suggesting a role for MARCKS in PDGF-BB induced motility. To determine if MANS peptide treatment inhibits PDGF-BB mediated fibroblast migration, a Boyden chamber approach was utilized with fibronectin-coated transwells and PDGF-BB or vehicle control (VC; sterile water) placed in the bottom chamber. A concentration of 1 nM PDGF-BB was used for these studies as we have previously demonstrated this concentration to be optimal for inducing directional chemotaxis in NIH-3T3 fibroblasts [31]. As shown in Figure 3, pretreatment of cells with the MANS peptide attenuated fibroblast migration towards PDGF-BB compared to RNS peptide or PBS (VC) treatment. Similar to the scratch wounding assays, MANS peptide inhibition of
migration was comparable to treatment with 100 nM wortmannin (Figure 3). Interestingly, unstimulated migration in wells containing VC was not affected by MANS treatment, suggesting that MARCKS protein specifically regulates directed migration of fibroblasts.

**MANS peptide reduces fibroblast chemotaxis as monitored by live-cell imaging**

To further support the conclusion that MANS peptide treatment inhibits MARCKS function and attenuates fibroblast chemotaxis, we used live-cell imaging by total internal reflection fluorescence (TIRF) microscopy. NIH-3T3 fibroblasts expressing GFP-AktPH, a fluorescent biosensor for PI3K signaling, were used to simultaneously monitor cell movement and polarity of intracellular signaling in response to PDGF-BB gradients [31]. Whereas cells treated with RNS control peptide exhibited normal morphology and migration response, cells treated with MANS peptide tended to exhibit smaller contact areas and reduced motility (Figures 4A & B). Chemotactic fidelity was quantified as the fraction of time the cell was moving towards the PDGF gradient (angle within 60°) less the fraction of time moving away from the gradient (angle between 120° and 180°); by this measure, the population of cells treated with MANS was underrepresented in cells exhibiting high fidelity (Figure 4C). Interestingly, the MANS-treated population was also underrepresented in cells moving predominantly away from the gradient, suggesting a general defect in cell migration persistence. Further analysis showed that, among the cells exhibiting the highest chemotactic fidelity in each population, the MANS-treated cells exhibited markedly less displacement from their original starting positions (Figures 4D & E). Interestingly, there were no differences observed in PI3K signaling as measured by the GFP-AktPH biosensor in either
MANS or RNS treated cells. Taken together, these results confirm our Boyden chamber experiments (Figure 2) and demonstrate that MARCKS is involved in the directional migration of NIH-3T3 fibroblasts.

**MANS peptide does not alter PDGF-BB-induced MARCKS or AKT phosphorylation**

As previously stated, PDGF-BB results in phosphorylation of MARCKS in Swiss 3T3 fibroblasts [34-36] as well as in human hepatic stellate cells [10]. Given that phosphorylation of MARCKS and subsequent membrane to cytosolic translocation is associated with MARCKS function, we asked if MANS peptide inhibition of PDGF-BB mediated chemotaxis was due to altered MARCKS phosphorylation. To address this question, we performed initial experiments to determine the conditions for optimal PDGF-BB induced MARCKS phosphorylation in fibronectin adherent NIH-3T3 fibroblasts. First, we performed a dose response assay by stimulating cells with 100, 10, 1 or 0.1 nM PDGF-BB for 1 minute and found 10 nM to be the optimal concentration of PDGF-BB to stimulate MARCKS phosphorylation (Figure 5A). Next, we performed a kinetics analysis by stimulating cells with 10 nM PDGF-BB for 1, 5, 10 or 20 minutes and found a 1 minute stimulation with 10 nM PDGF-BB resulted in optimal MARCKS phosphorylation (Figure 5B). To address if MANS peptide treatment alters PDGF-BB induced MARCKS phosphorylation, adherent NIH-3T3 fibroblasts were pretreated with MANS, RNS or PBS (VC) for 30 min and then stimulated with 10 nM PDGF-BB for 1 min. As shown in Figure 5C, MANS pretreatment did not alter MARCKS phosphorylation in either VC or PDGF-BB stimulated NIH-3T3 cells and no difference in MARCKS phosphorylation was observed in
MANS, RNS or PBS treated cells. This demonstrates that MANS peptide inhibition of PDGF-BB induced fibroblast motility is not due to alterations in MARCKS phosphorylation.

To address if MANS peptide treatment interferes with PDGF-BB mediated PI3K signaling, we evaluated phosphorylation of Akt in cells stimulated with PDGF-BB that were treated with MANS or RNS. As shown in Figure 5C, no differences in Akt phosphorylation were observed in MANS, RNS or PBS treated cells upon PDGF-BB or VC stimulation. These results confirm the results of the GFP-AktPH biosensor live-cell imaging experiment (Figure 4) and demonstrate that MANS peptide treatment does not interfere with PDGF-BB induced PI3K signaling.

*Expression of MARCKS is not required for NIH-3T3 fibroblast migration*

To address if MANS peptide treatment affects migration of NIH-3T3 fibroblasts in a similar manner as MARCKS knockdown, we transfected cells with two separate siRNAs targeting MARCKS and performed scratch-wounding assays on a fibronectin substrate. As shown in Figure 6A, we observed decreased protein expression of MARCKS 48 hours after transfection in cells that were administered either MARCKS A siRNA or MARCKS B siRNA. No difference in MARCKS protein expression was observed in control siRNA or non-treated cells and equal expression of beta-actin was observed in non-treated cells as well as control and MARCKS siRNA treated cells. As shown in Figure 6B, no difference in cell migration was observed in non-treated cells or cells transfected with control siRNA, MARCKS A siRNA or MARCKS B siRNA, suggesting that expression of MARCKS is not essential for migration of NIH-3T3 fibroblasts. We next stimulated control siRNA,
MARCKS A siRNA or MARCKS B siRNA transfected cells with MANS peptide to address whether MANS peptide treatment alters cell migration in these cells. As shown in Figure 6B, while MANS peptide treatment attenuated migration in control siRNA transfected cells, no difference in cell migration was observed in MARCKS A siRNA or MARCKS B siRNA transfected cells that were treated with or without MANS peptide. Similarly, reduced cell spreading was observed in control siRNA transfected cells treated with MANS peptide, whereas normal spreading pattern was observed in MARCKS siRNA transfected cells with or without MANS peptide treatment (data not shown). Further, no difference in cell proliferation was observed within the parameters of the scratch assay in control siRNA, MARCKS A siRNA or MARCKS B siRNA transfected cells with or without MANS peptide treatment (data not shown). Taken together, this data demonstrates that MARCKS is not essential for NIH-3T3 fibroblast migration and further demonstrates that the MANS peptide specifically targets MARCKS in NIH-3T3 fibroblasts.

Myristoylation of MANS is not required for inhibition of fibroblast migration

The MANS peptide is identical to the amino-terminus of MARCKS, including the presence of a myristic acid moiety. Whether or not myristoylation, which can enhance cell permeability and contribute to membrane attachment, is required for MANS attenuation of fibroblast migration was addressed using a genetic structure-function approach. We generated pEGFP-N1 expression plasmids encoding C-terminal EGFP fusion proteins of either the MANS sequence or a G2A point mutant that eliminates the myristoylation signal, resulting in unmyristoylated MANS (UMANS). The parent pEGFP-N1 vector served as a
control plasmid for these experiments rather than RNS::EGFP. Since RNS is a myristoylated peptide with the same amino acid composition as MANS but in a random scrambled sequence, cloning the RNS sequence into pEGFP-N1 using traditional methods would be challenging. Additionally, myristoyl-CoA:protein N-myristoyltransferase myristoylates proteins at the amino acid consensus sequence is NH2-GXXXS. While both MANS and RNS peptides are commercially synthesized with an amino-terminal myristic acid, it is likely that only MANS would be myristoylated upon epigenetic expression as it has the sequence NH2-GAQFS. RNS, with a sequence of NH2-GTAPA, would likely not be myristoylated upon epigenetic expression as it lacks a serine in the in the fifth position of the myristoylation consensus sequence [37,38]. Thus, we chose to use the parent pEGFP-N1 vector for the control of these experiments as there is a chance that RNS::EGFP would likely function differently than the RNS peptide.

Western blot analysis using an anti-EGFP antibody demonstrated equal expression of EGFP, MANS::EGFP and UMANS::EGFP proteins 24-hours post transfection (Figure 7A). Western blot analysis also revealed equal expression of MARCKS in non-transfected, EGFP, MANS::EGFP and UMANS::EGFP transfected cells (Figure 7A). Subcellular fractionation revealed that MANS::EGFP appears to be predominantly targeted to the membrane fraction (with some cytosolic localization) while EGFP and UMANS::EGFP are predominantly localized to the cytosol (Figure 7B). Interestingly, in spite of the fact that UMANS::EGFP does not contain a myristoylation signal, the fusion protein did appear to associate with the membrane fraction, albeit less so than MANS::EGFP. As a control for fraction purity and
equal protein loading, the expression of p38 MAPK (cytosolic fraction only) and beta-actin were also determined, respectively.

We next determined whether expression of MANS::EGFP affected fibroblast migration in a similar manner to MANS peptide treatment. Evaluation of NIH-3T3 cell migration using the scratch-wounding assay demonstrated that expression of MANS::EGFP significantly decreased migration compared to non-transfected cells and cells expressing EGFP alone. Further, expression of MANS::EGFP attenuated migration to a level approximately equal to that of 50 μM MANS peptide treatment (Figure 8A). Interestingly, expression of UMANS::EGFP also significantly inhibited NIH-3T3 fibroblast migration, similar to MANS peptide treated cells or cells expressing MANS::EGFP (Figure 8A). Expression of either MANS::EGFP or UMANS::EGFP in NIH-3T3 cells significantly inhibited migration in response to PDGF-BB in Boyden chamber chemotaxis assays compared to untreated cells and cells expressing EGFP alone (Figure 8B). As in the scratch assay, expression of MANS::EGFP and UMANS::EGFP fusion proteins inhibited PDGF-BB mediated fibroblast migration comparably to 50 μM MANS peptide treatment. There was no difference in unstimulated migration in non-treated, MANS treated or transfected (EGFP, MANS::EGFP or UMANS::EGFP) cells. These results confirmed that the MANS peptide, whether delivered by a cell permeable peptide approach or by epigenetic expression, significantly inhibits fibroblast migration. This data further demonstrates that myristoylation is not required for the ability of the MANS peptide to inhibit fibroblast migration and that other aspects of the MANS peptide, related specifically to the amino acid sequence, are involved in regulating MARCKS function related to fibroblast migration.
Discussion

Herein, a role for MARCKS in regulation of NIH-3T3 fibroblast migration was demonstrated. The MANS peptide, a myristoylated peptide corresponding to the first 24-amino acids of MARCKS that has been shown to inhibit MARCKS function, decreased migration of NIH-3T3 cells in scratch-wounding, PDGF-BB transmembrane chemotaxis assays and live-imaging chemotaxis assays. This finding is in accordance with previous studies from this laboratory demonstrating a role for MARCKS in migration of neutrophils [16] and mesenchymal stem cells [17]. Given these results, we hypothesize that the amino-terminus of MARCKS is required for regulating MARCKS function during fibroblast migration, given that either the MANS peptide or epigenetically expressed MANS similarly attenuated NIH-3T3 migration. Further experiments are underway in our laboratory is necessary to address this hypothesis.

To address which aspects of the MANS peptide are involved in regulating fibroblast migration, we utilized a genetic structure-function approach to express MANS and unmyristoylated MANS (UMANS) C-terminal EGFP fusion proteins in NIH-3T3 cells. Similar expression of EGFP, MANS::EGFP and UMANS::EGFP was observed in transfected cells. Equal expression of MARCKS was also observed in non-transfected cells and cell transfected with either EGFP, MANS::EGFP or UMANS::EGFP. This was slightly surprising to us as previous reports demonstrate that transformed 3T3 cells express decreased levels of MARCKS relative to non-transformed cells due to transcriptional down-regulation [39-41].
Previously, we demonstrated that isolated neutrophils are permeable to both the MANS and RNS peptides, presumably because of the amino-terminal myristoyl moiety. Further, subcellular fractionation studies demonstrated that the MANS peptide displaces MARCKS from cell membranes in untreated neutrophils, whereas the RNS peptide allows for MARCKS to remain localized to the membrane [16]. Similarly, MANS peptide treatment resulted in displaced MARCKS binding from mucin granules in human bronchial epithelial cells, which correlated with decreased mucin secretion in asthma models [42]. Herein, we demonstrate that MANS::EGFP fusion proteins are preferentially targeted to the membrane fraction of NIH-3T3 fibroblasts, with some cytosolic localization. Conversely, EGFP and UMANS::EGFP are primarily localized in the cytosol, with some localization in the membrane fraction. These results indicate that myristoylated and unmyristoylated MANS localizes to cell membranes and may disrupt cell migration by competing with MARCKS for membrane binding sites within cells. It should be noted that the exact membrane that MANS::EGFP and UMANS::EGFP localizes to has yet to be determined.

Results of previous studies demonstrated that β2-integrin mediated neutrophil adhesion is regulated by MARCKS function although MARCKS does not affect expression of β2-integrins on neutrophils [16]. In the present study, a role for MARCKS in regulating fibroblast migration on both fibronectin and collagen substrates is demonstrated. Integrins that recognize collagen include α1β1 and α2β1 [43] while α4β1 and α5β1 are integrins that recognize fibronectin [44,45]. NIH-3T3 cells express both fibronectin and collagen integrins [44,46] and the results reported here suggest that MANS peptide mediated attenuation of NIH-3T3 migration occurs independent of specific integrins expressed by the cell.
Herein, we also demonstrate that MANS peptide treatment does not alter the proliferation of NIH-3T3 fibroblasts as there was no difference in proliferation of cells that were not treated or treated with MANS, RNS, PBS (VC) or wortmannin (Figure 2). This confirms that in the decreased migration observed in the scratch-wounding assay is due to altered cell migration and not cell proliferation. It is has been shown that MARCKS negatively regulates fibroblast proliferation as quiescent Swiss 3T3 cells express high levels of MARCKS mRNA and protein that are down regulated upon cell proliferation [47]; similar observations have also been observed in other cell types [48,49]. As shown in Figure 5, MANS peptide treatment does not alter MARCKS expression as equal MARCKS protein was observed in non-treated cells or cells treated with MANS, RNS or PBS (VC). Further, siRNA knockdown of MARCKS did not alter cell proliferation in comparison to non-treated or control siRNA transfected cells and MANS peptide treatment did not alter these observations (data not shown). Thus, while MANS may inhibit MARCKS function in regards to cell migration, MANS peptide treat does not alter MARCKS expression or cell proliferation within the parameters of an 18-hour scratch-wounding experiment. Further experimentation, which does not fall within the scope of this study, is needed to determine if MANS peptide treatment alters the proliferation of NIH-3T3 fibroblasts in experiments lasting longer than 18 hours.

To address if MANS peptide treatment or decreased protein expression of MARCKS results in similar levels of attenuated migration, we knocked down the expression of MARCKS by siRNA treatment (Figure 6). Using a scratch-wounding assay, we observed similar levels of migration in non-treated, control siRNA or MARCKS A or B siRNA treated
cells. Interestingly, Rombouts, *et al.* observed similar results as increased PDGF-BB chemotaxis occurred in human hepatic stellate cells (hHSCs) that were transfected with a siRNA targeting MARCKS. Further, decreased PDGF-BB chemotaxis was observed in hHSCs overexpressing MARCKS [10]. Thus, our data confirms that of Rombouts, *et al.*, and demonstrates that MARCKS is not essential for cell migration in NIH-3T3 fibroblasts. The results in Figure 6 also demonstrate that the MANS peptide specifically targets MARCKS during cell migration as MANS peptide treatment does not alter the migration of cells with decreased MARCKS protein expression. As previously stated, our lab has shown that the MANS peptide displaces MARCKS from the plasma membrane of neutrophils [16] and from mucin granules of human bronchial epithelial cells [42], resulting in decreased cell migration and mucin secretion, respectively. While these studies demonstrate that MANS specifically interferes with MARCKS membrane localization and presumably function, these studies do not rule out the possibility that MANS peptide treatment may also have off-target effects. Given that we did not observe any difference in cell migration in MARCKS A or B siRNA transfected cells that were also treated with the MANS peptide, we can conclude that the MANS peptide specifically interferes with MARCKS function and does not have any off-target effects. Further, from the experiments shown herein, we can conclude that while MARCKS may not be essential, the process of cell migration is regulated by the presence of functional MARCKS protein and inhibition of MARCKS function by MANS peptide treatment results in abrogated cell migration.

As previously stated, we have demonstrated that MANS peptide treatment of neutrophils and mesenchymal stem cells results in decreased chemotaxis [16,17].
Additionally, MARCKS phosphorylation and perinuclear translocation is observed in microglial cells stimulated by the chemoattractant amyloid beta protein, suggesting a role for MARCKS in microglial cell chemotaxis [50-52]. Further, modifications of MARCKS protein expression in hHSCs results in altered PDGF-BB chemotaxis [10]. Herein, we used a live-cell imaging approach [31] to show that MANS peptide treatment interferes with directional migration of fibroblasts. MANS peptide treated cells did not exhibit high fidelity of chemotaxis and had a lesser degree of displacement from their original starting position in comparison to RNS treated cells. This demonstrates that MARCKS functions downstream of the chemokine receptor, possibly assisting in remodeling the actin cytoskeleton and/or relaying signals from the chemokine receptor to integrins that are essential for cell migration.

One potential mechanism by which MARCKS acts to regulate cell migration is through the sequestration of phosphatidylinositol 4, 5-bisphosphate (PIP$_2$). The unphosphorylated effector domain of MARCKS binds to PIP$_2$-rich regions in the plasma membrane, thus clustering and sequestering PIP$_2$ molecules. This MARCKS mediated sequestration of PIP$_2$ has been shown to inhibit phospholipase C-gamma (PLC-gamma) mediated hydrolysis of PIP$_2$. Upon chemoattractant stimulation, MARCKS becomes phosphorylated or bound by CaM and dissociates from the plasma membrane, releasing its sequestration of PIP$_2$ and thereby allowing PLC-gamma to hydrolyze PIP$_2$ [53-55]. Actin cytoskeletal dynamics are regulated by the concentration of free PIP$_2$, as the concentration of PIP$_2$ signals for anchoring or releasing the actin cytoskeleton from the plasma membrane [53,56,57]. Thus, PIP$_2$ acts to tightly regulate the process of cell migration by coordinated attachment and release of the actin cytoskeleton from the plasma membrane. PIP$_2$ is also
involved in talin activation, with talin activating and regulating integrin function that is essential to cell migration [58-60]. As described above, MANS peptide treatment displaces MARCKS from the plasma membrane in non-stimulated neutrophils [16] as well as from mucin granules in human bronchial epithelial cells [42], demonstrating that MANS competitively inhibits MARCKS binding to the plasma membrane. Given these observations, we hypothesize that the mechanism by which MANS peptide interferes with directed cell migration could be through lack of MARCKS mediated PIP$_2$ sequestration, resulting in the constant availability of PIP$_2$ to be hydrolyzed by PLC-gamma. Additional studies are underway in our laboratory to address this hypothesis.

Previous work has demonstrated that unmyristoylated MARCKS preferentially localizes to the cytosol, with some localization to the membrane, and it is thought that low-level membrane localization of unmyristoylated MARCKS is due to weak electrostatic interactions between the effector domain of MARCKS and the plasma membrane [3,13,61]. Interestingly, myoblasts transfected with unmyristoylated MARCKS were still capable of adhesion and spreading on a fibronectin substrate while MARCKS effector domain deletion mutants were not, suggesting that myristoylation of MARCKS is not essential to its function [13]. Given this, we can be fairly certain that unmyristoylated MARCKS would likely not affect cell migration as spreading are key steps to the process of cell motility.

Results of the studies reported here indicate that myristoylation of the MANS peptide itself is not required for MANS peptide-mediated inhibition of MARCKS regulated fibroblast migration. Epigenetically expressed unmyristoylated MANS (UMANS::EGFP), which was localized to both the membrane and cytosol, was capable of inhibiting cell
migration in a similar manner to MANS peptide treatment or epigenetic expression of MANS::EGFP. These findings demonstrate that myristoylation of the MANS peptide is not essential for inhibition of cell migration. This data further suggests that membrane localization of the MANS peptide is not essential to disrupting MARCKS function and other aspects of the MANS peptide may be involved. There are specific amino acids within the MANS peptide (or the amino-terminus of MARCKS) that could potentially be involved in regulating MARCKS function. Candidate amino acids within the amino-terminus of MARCKS that may be involved in regulating cell migration are Lys\(^6\) and Thr\(^7\), as it is known that proteolytic cleavage occurs at this site by an unidentified protease [62]. Calpain, a potential candidate for the unidentified protease [63,64], is a calcium-activated protease that is localized to the leading edge of polarized neutrophils and is involved in pseudopod formation and chemotaxis [65,66]. It is also involved in lymphocyte function-associated antigen-1 (LFA-1) mediated T-lymphocyte adhesion as well as focal adhesion formation in bovine aortic endothelial cells [67,68]. Calpain is known to cleave MARCKS in myoblasts, resulting in a 55 kDa fragment [63] and inhibition of calpain activity resulted in decreased myoblast migration associated with an accumulation of membrane bound MARCKS [63,69-71]. Further, adenosine triphosphate (ATP)-mediated activation of calpain results in amino-terminal MARCKS cleavage products in virally transformed human bronchial epithelial cells (HBE-1) [72]. Thus, one could propose that generation of the six amino acid fragment by proteolytic cleavage of MARCKS between Lys\(^6\) and Thr\(^7\) may be involved in regulating cell migration, with further experimentation needed to confirm this hypothesis.
PDGF-BB, a known mitogen and chemoattractant for fibroblasts, signals through a receptor tyrosine kinase, PDGFR. Signaling through PDGFR increases intracellular Ca\(^{2+}\) concentrations and activates PKC, both of which mediate MARCKS function[30,73]. In Swiss 3T3 cells, PDGF-BB stimulation results in phosphorylation of MARCKS and subsequent membrane to cytosol translocation [34-36]. MARCKS can be phosphorylated by PKC\(\alpha\), PKC\(\varepsilon\) and PKC\(\theta\) isoforms in NIH-3T3 cells[74] and PDGF-BB is known to activate PKC-\(\alpha\) in various fibroblast lines[75,76]. PDGF-BB mediated MARCKS phosphorylation is dependent on both PKC-\(\alpha\) and \(-\varepsilon\) during the migration of hHSCs [10]. Thus, it is likely that PKC\(\alpha\) is involved in PDGF-BB induced MARCKS phosphorylation in the studies reported here, although identification of the exact PKC isoform(s) involved was not performed. Interestingly, MANS peptide treatment did not alter PDGF-BB mediated phosphorylation of MARCKS in adherent NIH-3T3 fibroblasts (Figure 5), demonstrating that MANS peptide mediated attenuation of fibroblast migration is not due to abnormalities in MARCKS phosphorylation.

Additionally, there were no observed abnormalities in PI3K activity in MANS or RNS treated cells, as measured by an EGFP-AktPH biosensor (Figure 4) or phospho-Akt Western Blot analysis (Figure 5). PDGF-BB signaling in fibroblasts results in PI3K localization to the polarized leading edge, and inhibition of PI3K activity results in attenuated cell migration [27,31]. Given that abnormal PI3K signaling in MANS treated cells was not observed, these results indicate that the MANS peptide-attenuated cell migration occurs independently of PI3K activation. However, PI3K signaling is known to be involved in PKC...
activation and subsequent MARCKS phosphorylation [77,78], so it would appear that while PI3K may affect MARCKS function, MARCKS does not appear to affect PI3K activity.

In summary, these studies have indicated an important role for MARCKS, specifically its amino-terminus, in regulating NIH-3T3 fibroblast migration. While the MARCKS-specific MANS peptide attenuated migration, myristoylation of MANS does not appear to regulate this process. Given that the MANS peptide has also been shown to inhibit neutrophil and mesenchymal stem cell migration [16,17], the results reported here provide further evidence for the concept that targeting MARCKS may be a valuable novel therapeutic approach for various diseases associated with exacerbated cell migration, as occurs in inflammation, injury/repair and metastatic disease.

Materials and Methods

Reagents and cell culture

MANS and RNS peptides were synthesized as previously described [38] and resuspended in sterile PBS. Wortmannin was obtained from Sigma (St. Louis, MO) and a stock solution was made in DMSO (Sigma). PDGF-BB and fatty acid free bovine serum albumin (BSA) were purchased from Sigma and were resuspended in sterile water or PBS, respectively. Type II rat-tail collagen and fibronectin (Sigma) were resuspended in 0.1% acetic acid v/v or sterile water, respectively.
NIH-3T3 fibroblasts (ATCC, Manassas, VA) were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA) and 0.2% Penicillin (10,000 U/ml) Streptomycin (10,000 μg/ml) solution (Gemini Bio-Products).

Recombinant plasmids and transfections

MANS and UMANS inserts were PCR amplified from pCDNA4/TO wt MARCKS plasmid with glycine (GGT) to alanine (GCT) point mutation in the second amino acid position for UMANS. MANS and UMANS were cloned into the EcoRI and BamHI restriction sites of pEGFP-N1 (Clonetech, Mountain View, CA); colonies were screened by colony PCR using pEGFP-N1 sequencing primers and positive colonies were sequenced (MWG, Huntsville, AL). Qiagen’s EndoFree Maxi Kit (Qiagen, Valencia, CA) was used to prepare purified plasmid that was endotoxin free. NIH-3T3 fibroblasts were transfected by nucleofection using the Amaxa® Cell Line Nucleofector Kit® R (Lonza, Basel, Switzerland) following manufacturer’s protocol with 10 µg of plasmid per reaction. Transfected cells were plated in 6-well tissue culture plates and the expression of EGFP fusion proteins was determined by fluorescent microscopy or western blot analysis 18 hours after transfection; transfected cells were used for cell migration analysis within 24 hours.

siRNAs (Origene Technology, Rockville, MD) targeting the following sequences were used: 5’-GGAGTTCATGRAAACCATAGGAACT-3’ (MARCKS A), 5’-
GGAATGTAACGTTGCTTACAAGCAT-3’ (MARCKS B). Cells (1x10⁵) in a 6-well plate were transfected using Lipofectamine RNAiMAX reagent (Invitrogen) with 10nM of
MARCKS A, B or control siRNA. Gene knockdown was confirmed by western blotting 48 and 72 hours after transfection.

**Scratch Wounding Assay**

Fibroblast scratch assays were performed as described [79]. Briefly, sterile 22-mm coverslips (Fisher Scientific, Pittsburgh, PA) were coated with 10 µg/mL fibronectin or collagen in sterile 6-well tissue culture plates for two hours at room temperature. Coated coverslips were washed in sterile PBS and NIH-3T3 fibroblasts were seeded in complete media and cultured until confluent. For scratch assays with transfected cells, two nucleofection reactions per scratch were performed (1x10^6 cells/nucleofection or 2x10^6 cells/scratch assay) with scratch assays starting 18-24 hours after transfection. For scratch assays on siRNA knockdown cells, one siRNA reaction per scratch was performed with scratch assays occurring 48 hours after transfection. Two parallel scratches that were consistent in width were made in the monolayer using a standard sterile 200 µL pipette tip. The coverslips were washed with sterile PBS and replaced with DMEM containing 2% FBS and antibiotics. In some experiments, media was supplemented with MANS, RNS, sterile PBS (VC) or 100 nM wortmannin. The T=0 coverslip was immediately removed and processed prior to incubating the remainder of the plate for 18 hours at 37 °C, 5% CO_2. Coverslips were processed by fixing in 10% neutral buffered formalin solution (Fisher) and stained with Harris Hematoxylin or Diff-Quick following standard procedures. Coverslips were mounted onto microscope slides and an ocular micrometer was used to measure the wound distance at ten random locations along the scratch under a 40X objective. Wound
closure distance for each sample was determined by subtracting the average wound closure for each sample from the average initial T=0 wound distance with data represented as average percent wound closure ± standard error of the mean (SEM). Photographs of the scratches were obtained using a Nikon AZ100 microscope (Nikon, Melville, NY) under bright field conditions.

**CFSE Proliferation Assay**

NIH-3T3 fibroblasts were loaded with 2 μM CFSE (eBioscience, San Diego, CA) and plated (5x10^5 cells/well) on fibronectin (10 μg/mL) coated 6-well plates in complete media (DMEM with 10% FBS and antibiotics). Cells were allowed to adhere to the plate for 2 hours at 37°C, 5% CO₂ and then washed twice with sterile PBS. DMEM with 2% FBS and antibiotics was added to each well and cells were treated with either 50 μM MANS, 50 μM RNS, PBS (VC) or 100 nM wortmannin before incubating for 18 hours at 37°C, 5% CO₂. Cells were then harvested and fixed in 1% paraformaldehyde in PBS and cell proliferation was evaluated by flow cytometry using an Accuri C6 flow cytometer (30,000 total events collected) with data analysis performed using FloJo software.

**Transmembrane Chemotaxis Assay**

Fibroblast chemotaxis assays were performed as described [80]. Briefly, transwell inserts (8 μm pore size, 6.6 mm diameter; Corning, Corning, NY) were coated with 10 μg/mL fibronectin for two hours at room temperature and washed in PBS. Transfected or non-transfected NIH-3T3 cells were washed and resuspended in sterile serum-free DMEM at
a concentration of 5 × 10^5 cells/ml. In some experiments, cells were pretreated with 50 μM MANS, 50 μM RNS, sterile PBS (VC) or 100 nM wortmannin for 30 minutes at 37°C. Chemotaxis buffer consisted of serum free DMEM containing 1 mg/mL fatty acid free BSA; 1 nM PDGF-BB or VC (sterile water) was added to the chemotaxis buffer prior to addition to a 24-well plate with fibronectin coated transwells placed on top. NIH-3T3 cells (100 μL or 5 × 10^4 cells) were placed in the top chamber of each transwell and the plate was incubated for 4 hours at 37°C, 5% CO₂. Cells on the upper part of the filter were dislodged with a sterile cotton swab and rinsed with sterile PBS. Filters were fixed in 10% neutral buffered formalin solution and stained with harris hematoxylin or Diff-Quick prior to removing the filters from the transwell and mounting on glass microscope slides. The number of cells on the bottom of the filter was counted in 10 randomly selected high-powered fields (400X) of a light microscope.

*Western blotting and PDGF stimulation*

For PDGF stimulation of adherent cells, NIH-3T3 fibroblasts were seeded and grown to 90% confluence on 6-well plates that were coated with 10 μg/mL fibronectin. Cells were serum starved for 4 hours in serum-free DMEM plus antibiotics and 1 mg/mL fatty acid free BSA. In some experiments, cells were pretreated with 50μM MANS, RNS or VC (PBS) for 30 minutes at 37°C and cells were stimulated with PDGF-BB for the indicated time and plates were immediately placed on ice. Cells were washed with ice cold sterile PBS prior to the addition of 500 μL of RIPA buffer containing protease inhibitors (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM sodium pyrophosphate and
50 mM sodium fluoride, 1 mM phenylmethanesulphonylfluoride (PMSF) and 1:100 dilution of Sigma protease inhibitor cocktail) and were removed from the plate by scraping. Lysates were prepared by standard procedure and protein concentrations were determined by the BCA assay (Pierce, Rockford, IL). Boiled samples, diluted in 5X Laemmlli buffer, were separated by 10% SDS-PAGE. Phospho-MARCKS (Ser 152/156), phospho-AKT1 (Ser 473), total AKT1 and beta-actin antibodies were purchased from Cell Signaling Technology (Danvers MA) and total MARCKS (N-19 or M-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Western Blots were developed by ECL enhanced chemiluminescence (Thermo Scientific, Rockford, IL) and exposed to radiographic film.

**Subcellular Fractionation**

Subcellular fractionation was performed using the Subcellular Protein Fractionation Kit (Thermo Scientific) 24 hours after transfection according to the manufacturer’s instruction. Equal protein concentrations were loaded onto a 12% SDS-PAGE and Western blots were performed with the following antibodies: EGFP polyclonal (Santa Cruz Biotechnology), p38 MAPK (α, β, γ isoforms) and beta-actin (Cell Signaling). Western Blots were developed by ECL enhanced chemiluminescence and exposed to radiographic film.

**Live-cell fluorescence microscopy and image analysis**

Stable expression of the 3’ phosphoinositide-specific biosensor construct EGFP-AktPH in NIH 3T3 cells was established by retroviral infection as described previously [27]. These cells were plated on glass coated with human plasma fibronectin (10 mg/mL coating
concentration, obtained from BD Biosciences (San Jose, CA). The imaging buffer was 20 mM HEPES pH 7.4, 125 mM NaCl, 5 mM KCl, 1.5 mM MgCl₂, 1.5 mM CaCl₂, 10 mM glucose, and 2 mg/mL fatty acid-free bovine serum albumin, supplemented with 1% v/v fetal bovine serum. Localization of PI3K signaling was monitored by total internal reflection fluorescence (TIRF) using a prism-based microscope described in detail previously [27,33]. Chemotaxis experiments using alginate microspheres (a kind gift from Darrell Irvine, MIT) were carried out essentially as described [31], except that RNS or MANS peptide was added to the cells 30 minutes prior to the addition of the microspheres. Calculations of each cell’s centroid coordinates and the angle of its movement relative to the estimated PDGF gradient were performed as described [31].

Statistical analysis

Statistical analysis was performed by Sigma Stat Software (Systat Software, Inc, Chicago, IL) using Student’s t-test with P-values less than or equal to 0.05 considered statistically significant.
Figure 1. MANS peptide treatment attenuates migration of NIH-3T3 fibroblasts (performed by Dr. Laura Ott). NIH-3T3 fibroblasts were grown to confluency on fibronectin (A&B) or collagen (C) coated coverslips and scratch assays were performed with increasing concentrations (1, 50 or 100 μM) of MANS or RNS, VC (PBS) or 100 nM wortmannin. (A) Photos are representative of four separate experiments on fibronectin-coated coverslips; bar is equivalent to 500 μm in length. The average percent wound closure is shown on fibronectin (B) or collagen substrates (C), with four individual experiments for both substrates performed. Statistical analysis (p<0.05) was performed where “a” denotes a significantly decreased ability to migrate back into the wound relative to no treatment and “b”, “c”, and “d” denote a statistically significant ability to migrate back into the wound relative to 1 μM, 50 μM and 100 μM RNS, respectively.
(Studies shown in Figure 1 were performed by Dr. Laura Ott)
(Studies shown in Figure 2 were performed by Dr. Laura Ott)

Figure 2. Fibroblast proliferation is not altered by MANS peptide treatment

(performaed by Dr. Laura Ott). CFSE labeled NIH-3T3 fibroblasts (5x10^5 cells) were plated on fibronectin coated plates and allowed to adhere. Cells were incubated in DMEM with 2% FBS plus antibiotics in the presence of wortmannin (WORT; 100 nM), PBS (VC), 50 μM MANS or 50 μM RNS for 18 hours and cell proliferation was evaluated using flow cytometry. The gate displayed is the percentage of proliferating cells within a forward versus side scatter fibroblast gate and data is representative of three independent experiments. NT denotes CFSE-labeled cells that were not treated.
PDGF-BB mediated NIH-3T3 fibroblast chemotaxis is inhibited by MANS pretreatment (performed by Dr. Laura Ott). NIH-3T3 fibroblasts were pretreated with 50 μM MANS, 50 μM RNS, PBS (VC) or 100 nM wortmannin (WORT) for 30 minutes prior to adding the cells to fibronectin coated transwells with 1 nM PDGF-BB or VC (sterile water) in the bottom chamber. Transwell chambers were incubated for 4 hours and the transwell inserts were fixed, stained and mounted on microscope slides and the number of cells on the bottom side of the filter was counted in 10 high-powered fields. Data shown represents the average number of cells in 10 high-powered fields from four individual experiments with “a” and “b” denoting a significant decrease in percent (%) wound closure relative to RNS and VC treatment, respectively ($p<0.05$).
Figure 4. MANS inhibition of chemotaxis analyzed by live-cell TIRF microscopy (performed by Adam T. Melvin). (A) Time-course montage of a GFP-AktPH-expressing NIH 3T3 mouse fibroblast migrating chemotactically in response to a PDGF-BB gradient emanating from an alginate microsphere (open circle). The cell was treated with RNS peptide and monitored by TIRF microscopy, with localization of PI3K signaling displayed using a pseudo-color intensity scale; scale bar = 100 μm. (B) Montage as in part A, except that incubation was in the presence of MANS peptide, which inhibited productive cell movement. Multiple microspheres in this field are indicated by filled circles; scale bar = 100 μm. (C) Chemotactic fidelity was quantified as the fraction of time intervals during which cell movement was aligned with the PDGF gradient (angle within 60°) minus the fraction of time intervals during which movement was misaligned (angle between 120-180°). The histogram compares cells incubated with RNS (n = 32) versus MANS (n = 27) at peptide concentrations of 10 μM (gray, yellow) and 50 μM (black, red). (D&E) For each of the 4 treatment conditions compared in part C, chemotactic fidelity values were sorted into high, medium, and low subpopulations for RNS (D) and MANS (E). Cell centroid translocation paths are plotted with the initial centroid positions located at the origin and the initial PDGF-BB gradient vector aligned along the positive x-axis.
(Studies shown in Figure 4 were performed by Adam T. Melvin)
Figure 5. MANS pretreatment does not alter PDGF-BB mediated MARCKS phosphorylation (performed by Dr. Laura Ott and Eui Jae Sung). Adherent fibroblasts were stimulated with the indicated concentrations of PDGF-BB for the indicated times and Western Blot analysis for phosphorylated MARCKS (p-MARCKS) and total MARCKS (MARCKS) were performed. (A) Representative dose-response (1 min stimulation) studies from three separate experiments. (B) Representative kinetics analysis assays (10 nM PDGF-BB) from three separate experiments. (C) Adherent fibroblasts were pretreated with 50 μM MANS, 50μM RNS or PBS (VC for MANS or RNS peptides) for 30 minutes prior to stimulation with 10 nM PDGF-BB or VC (sterile water) for 1 minute. Western blot analysis was performed to determine the expression of phosphorylated MARCKS (p-MARCKS), total MARCKS (MARCKS), phosphorylated AKT1 (p-AKT), total AKT (AKT) and beta-actin. NT denotes no treatment and VC denotes vehicle control (sterile water); data is representative of three separate experiments.
Studies shown in Figure 5A and B were performed by Dr. Laura Ott.
Figure 6. MARCKS protein expression is not essential to NIH-3T3 fibroblast migration (performed by Eui Jae Sung). (A) NIH-3T3 cells were transfected with control, MARCKS A or MARCKS B siRNAs and total MARCKS and beta-actin expression was determined by Western blot analysis 48 hours post transfection. (B) Scratch-wounding assays on non-treated (not transfected), control siRNA, MARCKS A siRNA or MARCKS B siRNA transfected cells were performed with or without 50 μM MANS treatment. Statistical analysis ($p<0.05$) was performed where “a” denotes a significantly decreased ability to migrate back into the wound relative to no treatment and “b” denotes significantly decreased ability to migrate back into the wound relative to control siRNA transfected cells. Data is representative of three independent experiments with NT denoting no treatment.
Figure 7. Expression and cellular localization of MANS::EGFP and UMANS::EGFP fusion proteins (performed by Eui Jae Sung). (A) Transfection of EGFP, MANS::EGFP and UMANS::EGFP results in similar expression of EGFP in NIH-3T3 fibroblasts as determined by Western Blot analysis, with no expression of EGFP in non transfected cells. Total MARCKS expression in transfected and non-transfected NIH-3T3 fibroblasts was also determined by Western Blot, with beta-actin loading control. (B) Analysis of membrane and cytosolic fractions for the expression of EGFP, MANS::EGFP and UMANS::EGFP as determined by Western Blot analysis for EGFP expression. Fraction purity was determined by probing for the cytosolic marker p38 MAPK and beta-actin served as a loading control. Figures are representative of three independent experiments.
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- EGFP: 27 kDa
- MARCKS: 60 kDa
- beta-actin: 43 kDa

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- EGFP: 27 kDa
- p38 MAPK: 38 kDa
- beta-actin: 43 kDa
Figure 8. Myristoylation of MANS is not required for attenuation of fibroblast migration (performed by Dr. Laura Ott and Eui Jae Sung). (A) Transfected (EGFP, MANS::EGFP or UMANS::EGFP) or non-transfected NIH-3T3 fibroblasts were plated on fibronectin coated coverslips and scratch assays were performed as described. (B) PDGF-BB chemotaxis assays were performed with NIH-3T3 fibroblasts that were non-transfected or transfected with EGFP, MANS:EGFP or UMANS:EGFP as described. For both experiments, non-transfected cells were treated with the MANS peptide (50 μM) as a positive control for inhibition and no treatment denotes non-transfected cells. For both experiments, “a” and “b” designate statistically significant differences relative to non-transfected or EGFP transfected cells, respectively ($p<0.05$) and figure is representative of five independent experiments.
Studies shown in Figure 8A were performed by Dr. Laura Ott.
REFERENCES


