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Slit/Robo Signaling: Inhibition of Directional Leukocyte Migration

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1. Introduction

Localized inflammation and the associated influx of leukocytes is a hallmark of the pathogenesis of many diseases. The ability to target the recruitment of leukocytes holds vast therapeutic potential in inflammatory diseases where there is excessive cell recruitment due to an overactive immune response, or the improper resolution of the initial response resulting in chronic leukocyte infiltration.

1.1 The neutrophil

Polymorphonuclear leukocytes, or neutrophils, are a critical component of the innate immune system, participating in host defence against bacterial and fungal infections. Not surprisingly, neutropenias can lead to severe infections and sepsis. During an inflammatory response, neutrophils are recruited to the sites of infection and/or injury by chemoattractants, including the chemokine family of proteins. Once in the tissue, neutrophils fight infections by ingesting microorganisms and producing reactive oxygen intermediates (ROI) as well as other anti-microbial substances, such as defensins (Ganz, T., 2003). Neutrophils can also produce and/or exacerbate inflammatory disease states as a result of the potent systems that have evolved in these cells for microbial killing. Inappropriate or excessive activation of these systems results in tissue damage (Fujishima et al., 1995). To better understand the role of the neutrophil in the fine balance between host defence and tissue injury, the mechanisms underlying neutrophil recruitment will be discussed.

1.2 Neutrophils and tissue injury

Neutrophils have been implicated in the pathogenesis of several inflammatory conditions, including: ischemia reperfusion injury (following coronary artery occlusion) (Frangogiannis et al., 2002), idiopathic pulmonary fibrosis (Haslam et al., 1980), arthritis (Weissmann et al., 1984), asthma (Lemanske et al., 1983), vasculitis (Fauci et al., 1978), glomerulonephritis (Holdsworth et al., 1984) and acute respiratory distress syndrome (ARDS) (Wieland et al., 1999). Neutrophil-mediated tissue injury results from the release of neutrophil anti-microbial factors such as ROI and proteases, and other mediators that amplify cell recruitment into the extracellular milieu (Frangogiannis et al., 2001). This can occur in two ways: 1) activation of neutrophils leads to fusion of antimicrobial granules to the plasma

membrane and subsequent release of granule contents, and 2) attempts to ingest large particles result in a large open vacuole, and subsequent granule fusion and release of granule contents into the extracellular space (Weissmann et al., 1971).

ROI are strong oxidizing and reducing agents that damage the integrity of cell membranes by lipid peroxidation (Li et al., 2002). ROI also promote arachidonic acid synthesis by activating phospholipase A2. Arachidonic acid is an important precursor of eicosanoids and prostaglandins, including thromboxane A_2 and leukotriene B_4 (Toyokuni et al., 1999). Increased production of these pro-inflammatory molecules enhances recruitment of leukocytes. ROI also induce activation of transcription factors such as nuclear factor κB (NF-κB) and activator protein1 (AP1) (Toyokuni et al., 1999), leading to increased expression of adhesion molecules, including P-selectin, and chemokines (such as IL-8) thereby facilitating leukocyte arrest and recruitment from the circulation (Eltzschig et al., 2004).

Activated neutrophils also secrete matrix metalloproteases (MMPs), including collagenase and gelatinase. These enzymes are structurally specialized to digest basement membranes and interstitial structural proteins to facilitate neutrophil extravasation and subsequent migration through the interstitium (Kang et al., 2001). MMPs degrade several major structural components of the extracellular matrix (ECM), including collagen, fibronectin, proteoglycans, laminin and gelatin. MMPs are antagonized by tissue inhibitors of metalloproteases (TIMPs; Own et al., 1999). It has been shown that the imbalance between TIMPs and neutrophil-derived MMPs is a key feature of inflammatory conditions, including ARDS and asthma (Cederqvist et al., 2001). Neutrophil derived elastase is another bactericidal protease that is also associated with tissue damage. Like the MMPs, elastase displays proteolytic activity against structural components of the ECM. Elevated levels of neutrophil derived elastase and collagenase have been detected in patients with chronic inflammatory conditions, such as rheumatoid arthritis (Garcia et al., 1987). Increased neutrophil-derived protease activity has also been linked to cartilage destruction (Mohr et al., 1981). In ARDS, elastase activity has been associated with degradation of surfactant proteins in the lung (Hirche et al., 2004; Rubio et al., 2004). These proteins increase bacterial opsonization and clearance of apoptotic neutrophils (Vandivier et al., 2002). Therefore, increased elastase activity could indirectly increase susceptibility to infection and delay resolution of inflammation in the lung.

Commonly prescribed anti-inflammatory drugs, such as aspirin and glucocorticoids, have shown some success in reducing neutrophil-mediated tissue damage. However, these drugs generally attenuate activation of transcription factors such as NF-κB, thereby non-specifically reducing expression of cytokines and leukocyte adhesion molecules (Panes et al., 1999). One alternative method to prevent neutrophil-mediated tissue injury is to selectively block neutrophil recruitment to inflammatory foci. However, the redundancy in chemoattractant pathways means that interruption of a particular chemoattractant pathway may result in another pathway assuming its function. In principle, localized general chemoattractant blockade could be a useful strategy. Unique strategies to achieve this may be gained from studying central nervous system (CNS) development, in which positive and negative guidance cues for neuronal migration and axonal pathfinding have been defined.

1.3 Leukocyte trafficking and the adhesion cascade

The purpose of the inflammatory response is to selectively recruit the appropriate subsets of leukocytes to a site of inflammation. Inflammatory cytokines, such as interleukin 1 (IL-1)

and tumour necrosis factor α (TNF-α), and soluble chemoattractants, are released within the local inflammatory environment. This results in local vasodilation, increased volume of blood perfusing the inflamed area, and a simultaneous decrease in the flow velocity within the vessel, facilitating extravasation of circulating leukocytes. Leukocytes are recruited to sites of inflammation in a series of coordinated interactions with endothelial cells lining the vascular wall. The classical leukocyte adhesion cascade involves these main steps: i) leukocyte capture and rolling, ii) activation and arrest, and iii) transendothelial migration (Fig. 1). Failure in any one of these steps can result in severe immunodeficiencies (Beutler, B., 2004). However, there exists a substantial therapeutic potential for the localized blockade of leukocyte adhesion and diapedesis.

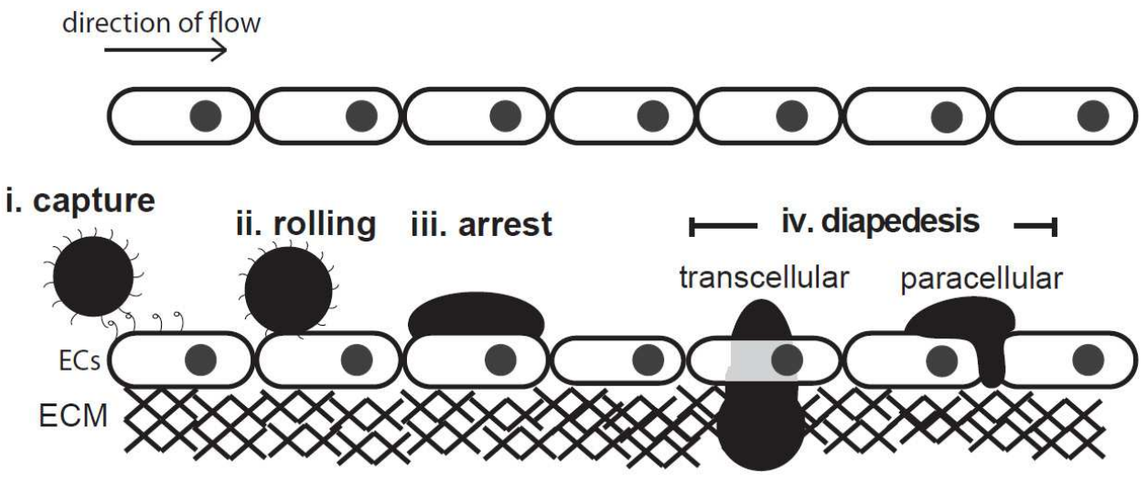

Fig. 1. Endothelial–leukocyte interactions leading to transmigration across the vascular wall. (i) Capture and (ii)rolling: The initial tethering of leukocytes to the endothelial cells lining the vessel wall is mediated by the selectins. These structural interactions enable the leukocyte to roll along the venular wall and to 'sample' the endothelial surface for activating factors (iii) Arrest: These interactions lead to leukocyte integrin activation. Firm adhesion of the leukocyte is mediated through binding of integrins to members of the immunoglobulin superfamily expressed in stimulated endothelial cells.(iv) Diapedesis: Following firm adhesion, the cell changes shape in response to local chemoattractant gradients and transmigrates across the endothelial barrier.

Selectins are a family of adhesion molecules that are structurally specialized for the initial capture of circulating leukocytes. Rolling is mediated by E-selectin and P-selectin, expressed by endothelial cells, and by L-selectin expressed on the majority of circulating neutrophils, monocytes, eosinophils, and T and B lymphocytes (Kansas, G., 1996). The broad expression pattern of L-selectin allows for nonspecific recruitment of all leukocyte lineages. P-selectin is constitutively found in Weibel-Palade bodies of endothelial cells, and mobilized to the cell surface within minutes following activation by inflammatory mediators (Frangogiannis et al., 2002). All of the selectins interact with P-selectin glycoprotein ligand 1 (PSGL1), although other glycoprotein ligands exist, such as CD34 and MadCAM-1 (McEver et al., 1997; Puri et al., 1995). Following initial leukocyte capture, the binding of leukocyte L-selectin to PSGL1 facilitates secondary leukocyte capture, where adherent leukocytes assist

in the recruitment of additional cells (Eriksson et al., 2001). Interactions of selectins with their ligands allow leukocytes to roll on inflamed endothelium under the rapid flow of the bloodstream (Alon et al., 1995). In fact, shear stress is required to support L-selectin and P-selectin dependent adhesion, and rolling cells detach when flow is stopped (Finger et al., 1996; Lawrence et al., 1997). This selectin-mediated slow rolling allows the leukocyte to 'sample' the repertoire of chemokines and other activation signals presented on the luminal surface of endothelial cells.

In addition to selectins, various integrins participate in rolling. Integrins bind members of the immunoglobulin superfamily, including vascular cell-adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1). Neutrophils roll on immobilized VCAM-1 by engaging the leukocyte integrin receptor, very late antigen 4 (VLA-4; $\alpha_4\beta_1$-integrin). β2-integrins also support rolling (Sigal et al., 2000). Resting mouse neutrophils roll on surfaces coated with E-selectin ligand and ICAM-1. Ligation of endothelial E-selectin induces a structural conformational change in leukocyte lymphocyte function-associated antigen 1 (LFA-1; $\alpha_L\beta_2$-integrin) allowing it to bind to its endothelial ligand, ICAM-1 (Salas et al., 2004). In addition, it has recently been demonstrated that the mechanochemical design of LFA-1 allows shear stress to induce and maintain a state of high ligand-binding affinity (Astrof et al., 2006). Rolling *in vivo* requires E-selectin (Kunkel et al., 1996), engagement of the β2-integrins (Jung et al., 1998), LFA-1 and macrophage antigen-1 (MAC1; Dunne et al., 2002).

Although leukocytes (particularly neutrophils) roll under normal conditions, during inflammation leukocytes undergo integrin-dependent arrest. Arrest of leukocytes on endothelial cells is rapidly triggered by the binding of chemokines and other chemoattractants (Campbell et al., 1998). These chemoattractants are secreted by activated endothelial cells and platelets. In fact, platelets can deposit chemokines, such as CC-chemokine ligand 5 (CCL5), CXC-chemokine ligand 4 (CXCL4), and CXCL5 onto the inflamed endothelial lumen to trigger leukocyte arrest (von Hundelshausen et al., 2001; Huo et al., 2003).

Following firm arrest, leukocytes migrate, by a process called diapedesis, across the endothelial cell barrier, its associated basement membrane, and the pericyte sheath. Leukocyte diapedesis and chemotaxis is triggered by chemokines (such as IL-8) presented to rolling leukocytes on the luminal surface of endothelial cells. Leukocytes can cross the endothelium between adjacent endothelial cells (paracellular route) or directly through an endothelial cell (transcellular route). Transcellular migration generally occurs in 'thin' parts of the endothelium where there is less distance for the leukocyte to migrate (Ley et al., 2007). In addition, caveolae containing ICAM-1 link together to form vesiculo-vacuolar organelles (VVOs), providing shortcuts for transcellular leukocyte diapedesis (Dvorak et al., 2001). This creates a channel inside the cell through which leukocytes can migrate. During paracellular migration, ligation of endothelial-cell adhesion molecules results in reduced interendothelial contacts, facilitating the migration of leukocytes through endothelial cell junctions (Ley et al., 2007). Transendothelial migration requires an increase in intracellular endothelial calcium, which promotes opening of endothelial cell junctions via the activation of myosin light chain kinase and endothelial cell contraction. The route of leukocyte migration is determined by both the surface density of ICAM-1 and the shape of endothelial cells (Yang et al., 2005). Both a high density of ICAM-1 and endothelial cells with a polygonal morphology promote transcellular migration (Yang et al., 2005). Many endothelial junctional molecules, such as platelet/endothelial-cell adhesion molecule 1 (PECAM-1), ICAM -1,

ICAM-2, junctional adhesion molecule A (JAM-A), (JAM-B), (JAM-C), endothelial cell-selective adhesion molecule (ESAM), and CD99, play a role in leukocyte transmigration. Although the leukocyte adhesion cascade has been divided into several steps, these are not temporally exclusive, but instead synergistically promote leukocyte arrest and diapedesis. Leukocyte diapedesis was described almost 200 years ago, but its molecular mechanisms are only now beginning to be more fully understood (Imhof et al., 2004). In the past decade, new insights have been gained into the signaling events that underlie integrin activation, post-adhesion strengthening of leukocyte attachment and the structural significance of molecules involved in diapedesis (Muller, W., 2003).

1.4 Chemotaxis

Following extravasation, leukocytes migrate through the interstitial ECM, following a chemoattractant gradient, to reach the site of inflammation. Chemotaxis, directed cell migration towards external chemical gradients, occurs in many eukaryotic cells including: free-living organisms, leukocytes (during inflammation), endothelial cells (angiogenesis), spermatocytes (fertilization) and neurons (neurogenesis) (Singer et al., 1986). Upon exposure to a chemoattractant the cell orients itself in the direction of locomotion along the chemoattractant gradient. Polarization results from preferential pseudopod extension towards areas of higher chemoattractant concentration (Zigmond, S., 1974). Efficient chemotaxis requires coordination between pseudopod formation at the leading edge of the cell, and uropod retraction at the trailing edge. During chemotaxis, neutrophils extend short surface protrusions called filopodia, or microspikes, which are membrane extensions of approximately 0.1-0.2 μm in diameter and up to 20 μm in length. These structures act as cellular tentacles and are supported by a core bundle of actin microfilaments (Mattila et al., 2008). In neutrophils, filopodia support thin sheets of membrane-enclosed cytoplasm, called lamellipodia. Lamellipodia contain actin filaments and a meshwork of myosin II-associated microfilaments. In neutrophils, the actin network within the lamellipodia, together with other structural and regulatory proteins, comprises the molecular motor which drives cell locomotion (Jones et al., 1998). This locomotory apparatus works against cell-to-substratum adhesions called focal contacts or focal adhesions. Focal adhesions are molecular structures that utilize integrins to link the myosin II-containing bundles of cytoplasmic microfilaments (called stress fibers) to proteins in the extracellular matrix (ECM) (Critchley et al., 1999). In neutrophils, integrin-mediated contacts to the ECM take two forms: focal complexes and podosomes. Focal complexes are structurally similar to focal adhesions but lack stress fibers (Allen et al., 1997), while podosomes are distinct circular structures that are only observed in cells of the myeloid lineage (DeFife et al., 1999; Correia et al., 1999; Linder et al., 2003). In this way, cytoskeletal rearrangement permits leukocytes to migrate toward chemoattractant gradients.

1.5 Chemoattractants

Many types of chemoattractant recruit leukocytes to inflammatory foci. These include bacterial components, leukotrienes, complement factors and chemokines. C5a, the first chemoattractant identified, is a cleaved product derived from complement component C5 (Shin et al., 1968). Bacterial products such as fMLP (N-formyl-methionyl-leucyl-phenylalanine) and other N-formylpeptides also act as chemoattractants that non-specifically recruit leukocyte subsets to inflammatory foci. An important family of

chemoattractants involved in leukocyte recruitment to inflammatory foci is a family of chemoattractant cytokines called chemokines. Chemokines constitute a large family of small peptides that are structurally similar and that bind to a family of seven transmembrane G-protein coupled receptors (Rossi et al., 2000). The specific expression, regulation, and receptor binding patterns of each chemokine determine their functional diversity. Most chemokines are structurally conserved to bind to glycosaminoglycans (GAGs) on the luminal surface of endothelial cells. This binding is required for leukocyte recruitment *in vivo*. Indeed, chemokines with mutations in their GAG binding domains can induce *in vitro* chemotaxis, but are unable to recruit leukocytes to the peritoneal cavity *in vivo* (Johnson et al., 2005).

The binding of chemoattractants to their receptors activates leukocyte integrins instantaneously by inside-out signalling mechanisms (Shamri et al., 2005). They rapidly regulate integrin avidity by increasing both integrin affinity (by a conformational change that results in increased ligand binding energy and a decreased ligand dissociation rate), and valency (the density of integrins per area of plasma membrane involved in adhesion, determined by expression levels and lateral mobility) (Laudanna et al., 2002; Constantin et al., 2000). Through these signaling mechanisms, chemokines work as powerful activators of integrin-mediated adhesion and leukocyte recruitment.

1.6 Intracellular signaling of chemoattractant receptors

Several neutrophil chemoattractants, particularly chemokines, interact with specific receptors on the plasma membrane, transducing signals by coupling to heterotrimeric G proteins. Heterotrimeric G proteins are composed of an α, β, and γ subunit. The α subunit is the GDP/GTP binding element. When bound to GDP, the α subunit interacts with the β and γ subunits to form an inactive heterotrimer complex. Chemoattractant binding induces a conformational change in the receptor, exchanging GDP for GTP on the α subunit. The α subunit then dissociates from the receptor, releasing the Gβγ complex. The free Gα and Gβγ subunits are then available to bind and activate target enzymes such as phosphatidylinositol 3-kinase (PI3K), phospholipase C (PLC), or adenyl cyclase (Fig. 2). These enzymes generate secondary intracellular messengers that initiate a cascade of signaling events that ultimately culminate in cytoskeletal rearrangement and leukocyte migration.

Ligation of chemoattractant receptors leads to the activation of four major signaling pathways (Fig. 2): PLC, PI3K, mitogen-activated protein kinases (MAPKs) and Rho guanosine triphosphatases (GTPases). Once the Gα subunit dissociates, the Gβγ complex activates PLC, which cleaves phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$) to generate inositol (1,4,5)-triphosphate (IP_3) and diacylglycerol (DAG). Generation of IP_3 leads to the mobilization of intracellular calcium stores from the endoplasmic reticulum, and together with DAG, activates protein kinase C (PKC) (Li et al., 2000). The activation and recruitment of PKC to the plasma membrane promotes changes in the actin cytoskeleton that facilitate and/or drive cell spreading and migration (Fig. 2).

A convincing role for PI3K in chemoattractant receptor signaling and chemotaxis has been established (Li et al., 2000; Sasaki et al., 2000; Hirsch et al., 2000; Servant et al., 2000; Jin et al., 2000). Although there are at least four Class I PI3K isoforms in mammalian cells (Vanhaesebroeck et al., 1999), only a single Class IB variant has been shown to interact with

chemoattractant receptors in leukocytes. The outcome of Class I PI3K activation is phosphorylation of membrane $PI(4,5)P_2$ by activated PI3K, generating $PI(3,4,5)P_3$ at the plasmalemma. The Gβγ complex also activates PI3Kγ, activating Src-family kinases and generating $PI(3,4,5)P_3$ from membrane $PI(4,5)P_2$ (Krugmann et al., 1999), resulting in the recruitment of Ras GTPases and subsequent activation of MAPK pathways (Fig. 2) (Kintscher et al., 2000). Although MAPK signaling pathways are involved in chemotaxis and adhesion, the most important biochemical events for cell polarization are the production of PIP_3 and activation of Rho GTPases at the leading edge of the cell.

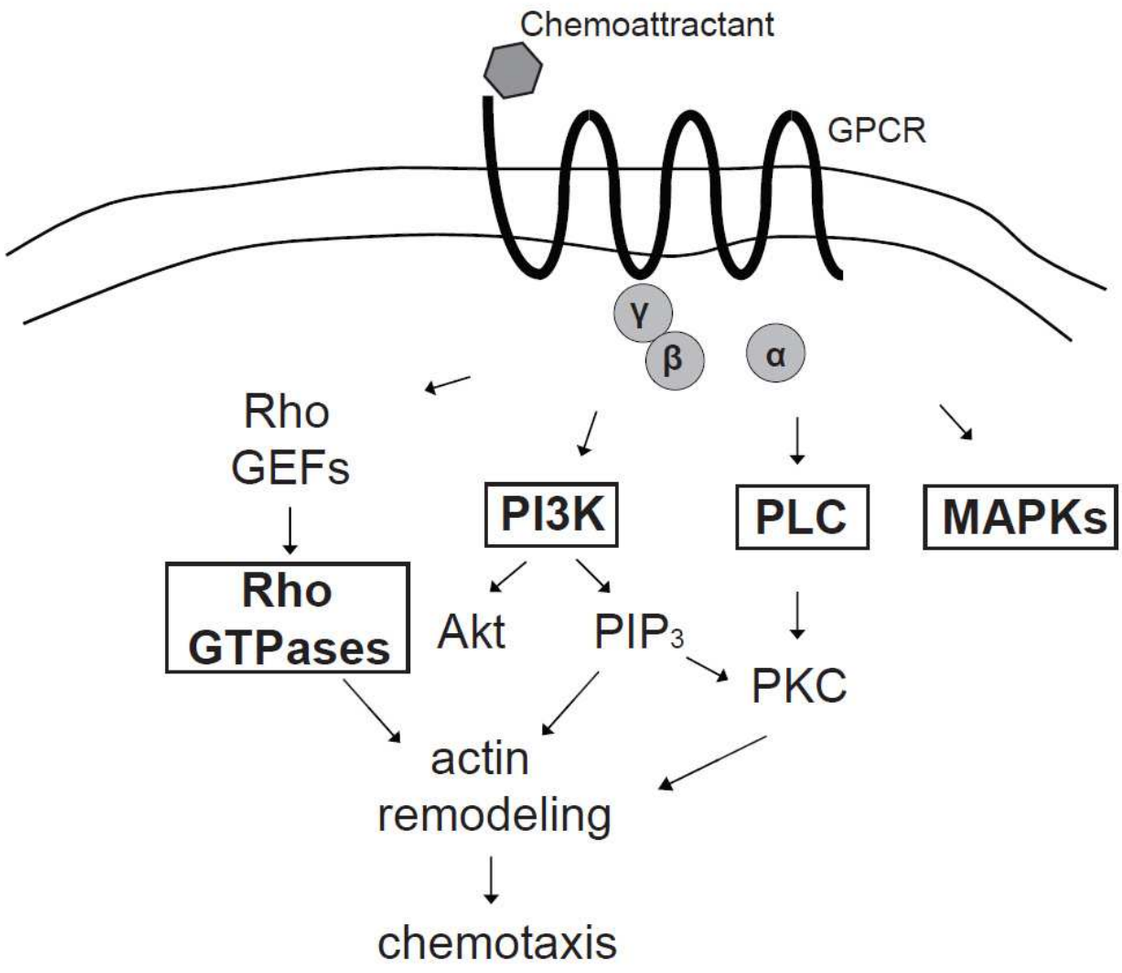

Fig. 2. Intracellular signaling cascade upon ligation of chemoattractant receptors. Chemoattractant binding to GPCRs induces a conformational change that results in the dissociation of Gα subunits from the Gβγ complex. This leads to rapid outside-in signaling resulting in the activation of four major signaling pathways that contribute to the generation of cell polarity and chemotaxis: Rho GTPases, PI3K, PLC, and MAPKs.

The PI3K dependent production of PIP_3 at the cell membrane allows for the recruitment of the Rho-family GTPases, Rac and Cdc42, to the cell membrane. The localization of PIP_3, Rac and Cdc42 then stimulate polymerization of actin, a process necessary for the formation of

filopodia and lamellipodia at the front of the cell. At the back of the cell, Rho-kinase phosphorylation results in inactivation of myosin light chain phosphatase, leading to increased myosin light-chain kinase (MLK) dependent activation of myosin (Nguyen et al., 1999). These biochemical conditions favour the formation of actomyosin bundles, contraction, de-adhesion from the substratum and tail retraction (Ridley, A., 2001; Bokoch, G., 2005). Interestingly, signals at the leading edge inhibit signals at the trailing edge, allowing for the maintenance of cell polarity (Fenteany et al., 2004). To prevent the accumulation of PIP_3 at the trailing edge, PTEN dephosphorylates $PI(3,4,5)P_3$ to $PI(4,5)P_2$. The lack of PIP_3 in the back of the cell prevents activation and recruitment of Rho GTPases and subsequent actin polymerization, allowing the formation of actomyosin bundles and tail retraction (Worthylake et al., 2001). Actin polymerization at the leading edge coupled with tail retraction in the back allows for directed leukocyte chemotaxis.

1.7 Rho-family GTPases: Rac, Cdc42, and Rho

Small GTPases of the Rho family are a part of the Ras superfamily of small GTP-binding proteins. They are pivotal regulators of many signaling networks that are activated by a diverse variety of receptor types. To date, over 20 mammalian Rho GTPases have been characterized, and these can be grouped into 6 different classes: Rac (Rac1, Rac2, Rac3, RhoG), Rho (RhoA, RhoB, RhoC), Cdc42 (Cdc42Hs, G25K, TC10), Rnd (Rnd3/RhoE, Rnd1/Rho6, Rnd2/Rho7), RhoD, and RhoH/translocation three four (TTF) (Aspenström, P., 1999; Kjoller et al., 1999). When activated, Rho GTPases regulate many important processes in all eukaryotic cells, including actin cytoskeleton dynamics, transcription, cell cycle progression, and membrane trafficking. The activity of Rho GTPases is regulated by outside-in signals from a variety of receptor types, including GPCR, tyrosine kinase receptors, cytokine receptors and adhesion receptors. Rho-family GTPases play a critical role in regulating leukocyte chemotaxis, adhesion and phagocytosis.

1.7.1 Rho GTPases: Structure and regulation

All Rho GTPases contain two main structural domains, the C-terminal 'CAAX' motif and a catalytic GTP domain. The 'CAAX' motif undergoes post-translational processing, involving carboxy-terminal proteolysis of the AAX residues followed by carboxyl-methylation. The modified C-terminal domain can then attach to membrane lipids and facilitates membrane association and subcellular localization of Rho GTPases (Gutierrez et al., 1989; Casey et al., 1989; Fujiyama et al., 1990). The catalytic domain contains two regions, switch I and switch II. These domains correspond to different structural conformations in the GTP-bound and GDP-bound forms. Rho GTPases function as molecular switches by cycling between GDP-bound and GTP-bound forms. When bound to GDP, Rho GTPases are inactive. Binding of ligands to cell surface receptors, results in exchange of GDP for GTP, switching the protein to an active state. The active form interacts with downstream effector molecules. The intrinsic GTPase activity of Rho GTPases completes this cycle by hydrolyzing GTP, returning the GTPase to its inactive GDP-bound state.

Three classes of molecules interact with Rho GTPases and regulate their activation state: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and

guanine nucleotide dissociation inhibitors (GDIs). GEFs catalyze the exchange of GDP for GTP, leading to the activation of Rho GTPases. To date, over 69 mammalian GEFs for Rho GTPases have been identified (Rossman et al., 2005). They are characterized by the presence of a Dbl homology domain (DH), which interacts with both the switch I and switch II regions and catalyses the exchange of GDP for GTP. In addition, many of these DH-domain containing proteins, such as Vav, contain a Pleckstrin homology (PH) domain which allows GEFs to bind phosphoinositides, such as PIP_3. This localizes GEFs to the plasma membrane where they can bind other Rho-family GTPase-interacting proteins. GAPs enhance the intrinsic GTPase activity of Rho GTPases, and thus suppress their activity. Although GTPases possess intrinsic GTPase activity, the actual rate of GTP hydrolysis is relatively slow. Therefore, the interaction with a GAP is required for efficient GTP hydrolysis, as this accelerates the cleavage step by several orders of magnitude (Vetter et al., 2001). To date, more than 70 eukaryotic RhoGAPs have been discovered, of which 35 are found in humans (Tcherkezian et al., 2007). There exists a large diversity in the primary sequences of the various GAPs. However, each one contains a Rho GAP domain with a conserved tertiary structure composed of α helices and a catalytically critical 'arginine finger' which stabilizes the formation of the transition state during GTP hydrolysis (Nassar et al., 1998). In addition, the Rho GAP domain interacts with both the switch I and switch II regions on the GTPase domain (Gamblin et al., 1998), allowing GAPs to facilitate the intrinsic hydrolysis of GTP, resulting in the inactivation of Rho GTPases.

Finally, GDIs associate with Rho GTPases in their inactive GDP-bound state and inhibit their activation by GEFs. GDIs also bind to GTP-bound GTPases, and suppress their activity (Oloffson, B., 1999). There is evidence that GDIs can bind to isoprenyl moieties on the C-terminus of GTPases in order to sequester them in the cytosol (Keep et al., 1997). The role of GDIs in partitioning GTPases between the membrane and cytosol may be physiologically more important than the inhibition of their activation, as this may provide a storage pool of Rho GTPases that is readily utilized upon cell activation. Overall, GDIs prevent the activation of Rho GTPases, prevent their interaction with membranes, and inhibit downstream signaling networks.

1.7.2 Rho GTPases and the actin cytoskeleton

The movement of eukaryotic cells relies on the coordinated extension of actin-rich lamellipodia in the leading edge and retraction of the uropod at the rear of the cell. The extension of lamellae in the leading edge involves rapid turnover of actin filaments (Symons et al., 1991; Wang, Y., 1985). More stable actin-myosin cables can be found in more established protrusions and in the middle and rear of the cell (DeBiasio et al., 1988). Recycling of the plasma membrane and integrin-mediated adhesion to the substratum and/or ECM are also important for cell motility (Bretscher, M., 1996; Martenson et al., 1993; Yamada et al., 1995; Mitra et al., 2005). Coordinated mobilization of the actin cytoskeleton is regulated by deployment of actin-binding proteins by activated Rho-family GTPases. Rho-family GTPases control cell motility and morphological changes in response to extracellular chemoattractants. Activation of Rho in fibroblasts results in the assembly of stress fibers and focal adhesions (Ridley et al., 1992). The activation of Rac causes extension of lamellipodia and assembly of small focal complexes (Nobes et al., 1995; Ridley et al., 1992). In contrast,

activation of the Cdc42 Rho-family GTPase leads to the formation of filopodial extensions (Nobes et al., 1995).

As discussed above, the influx of neutrophils and other leukocytes to inflammatory foci relies on activation of Rho-family GTPases and dynamic actin turnover. In principle, one method to prevent neutrophil-mediated tissue injury would involve blocking neutrophil recruitment. However, the redundancy in chemoattractant pathways means that interruption of a particular chemoattractant may result in another assuming its function. Thus, a localized general chemoattractant blockade could be a useful strategy. Unique strategies to target neutrophil recruitment may be gained from studying central nervous system (CNS) development, in which structurally distinct positive and negative guidance cues for migration and axonal pathfinding have been defined.

2. Slit2: A guidance cue for cell migration

During the development of the CNS, neurons must migrate and project axons over long distances. Most axons emanating from the CNS must cross the midline and then project longitudinally towards their synaptic targets. The molecular mechanisms that guide this pathfinding include contact attraction, chemoattraction, contact repulsion and chemorepulsion. Guidance cues selectively promote or repress migration of neurons and axonal projection. For example, netrins are diffusible chemotropic factors that attract commissural axons to the midline (Kennedy et al., 1994). The Slit family of secreted proteins, together with their cell-surface receptor Roundabout (Robo), repel neurons during CNS development. Once commissural axons have crossed the midline, midline glial cells express Slit to prevent axons from re-crossing the midline. Mutant Drosophila lacking Slit proteins exhibit midline defects, such as collapse of the regular scaffold of commissural and longitudinal axon tracts in the embryonic CNS (Rothberg et al., 1988; Rothberg et al., 1990). A similar defect is observed in mutant Drosophila lacking Robo, where projecting axon tracts cross the midline repeatedly (Kidd et al., 1998).

2.1 Slit and robo: Structure

The Slit family of proteins contain an N-terminal signal peptide, four leucine-rich repeats (LRRs), nine epidermal growth factor (EGF) repeats and a C-terminal cysteine knot (Fig. 3) (Rothberg et al., 1988; Rothberg et al., 1990; Rothberg et al., 1992). The EGF repeats and LRR allow Slit proteins to interact with ECM components, such as glypican-1, enabling them to act as localized, non-diffusible, signaling molecules (Ronca et al., 2001). Furthermore, Slit2 can be proteolytically cleaved after the fifth EGF repeat to form N-terminal (Slit2-N) and C-terminal (Slit2-C) fragments (Brose et al., 1999; Wang et al., 1999). Slit2-N includes the first 1118 amino acids and contains the four LRRs and the first five EGF repeats, while Slit2-C contains the remaining residues (Brose et al., 1999). Importantly, only the second LRR of human Slit2 is required to bind with the first Ig domain of Robo and initiate downstream signaling (Morlot et al., 2007). Therefore, both full length Slit2 and Slit2-N bind Robo receptors to repel migrating cells and projecting axons (Nguyen Ba-Charvet et al., 2001). Although the cleavage of Slit2 does not eliminate its activity, it may play a role in its diffusion since Slit-N appears to be more tightly associated with the cell membrane. In rat neural tissue both Slit2-N and Slit2-C were shown to bind heparan sulfate proteoglycan

glypican-1 (Liang et al., 1999), although Slit2-C bound with higher affinity, suggesting a possible regulatory mechanism for its diffusion.

Robo, a member of the immunoglobulin superfamily, is a single-pass type-1 receptor for the Slit proteins. The extracellular region of human Robo-1 contains five immunoglobulin (Ig) repeats and three fibronectin type III domains. The cytoplasmic region of Robo-1 contains four conserved cytoplasmic signaling motifs, CC0, CC1, CC2 and CC3 (Kidd et al., 1998; Zallen et al., 1998). Only the first Ig domain of Robo is required to bind to the second LRR domain in Slit2 and Slit2-N (Battye et al., 2001; Chen et al., 2001; Nguyen Ba-Charvet et al., 2001). The cytoplasmic CC motifs of Robo are required for response to Slit (Bashaw et al., 2000).

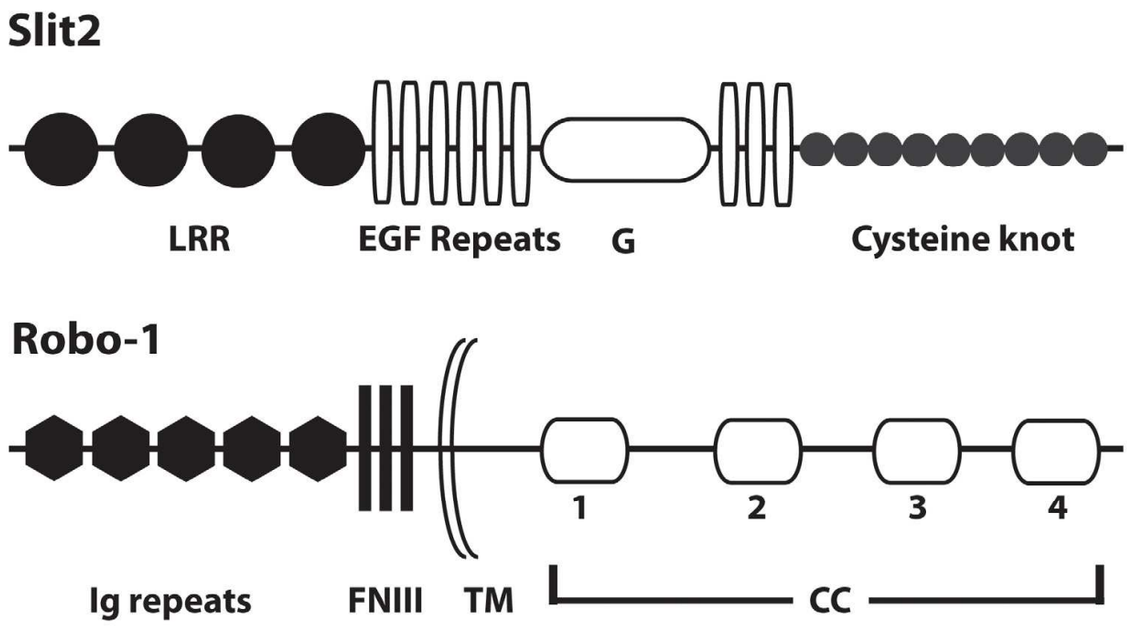

Fig. 3. Primary Protein Structure of Mammalian Slit2 and Robo-1 Proteins. Mammalian Slit2 contains four leucine rich repeats (LRRs), nine epidermal growth factor (EGF) repeats, a laminin G (G) domain, and a cysteine rich C terminus. The Robo-1 receptor contains five immunoglobulin (Ig) repeats, three fibronectin (FN) type III, a transmembrane Domain (TM) and four conserved cytoplasmic (CC) signaling motifs.

The detection of an amino-terminal fragment of Robo-1 (Robo-1-NTF) in the conditioned medium of cancer cell lines and in the serum of patients with hepatocellular carcinoma suggests that Robo-1 may undergo proteolytic cleavage (Ito et al., 2006). The cleavage site was recently shown to be between Glu852 and Glu853, only 10 residues away from the plane of the plasma membrane (Seki et al., 2010). Following cleavage of transmembrane Robo-1 by MMPs, a soluble Robo-1-NTF is generated. The remaining carboxy-terminal fragment (Robo-1-CTF1) is subsequently cleaved by γ-secretase to form Robo-1-CTF2 (Fig. 4). Robo-1-CTF2 translocates to the nucleus, although its function is unknown (Seki et al., 2010).

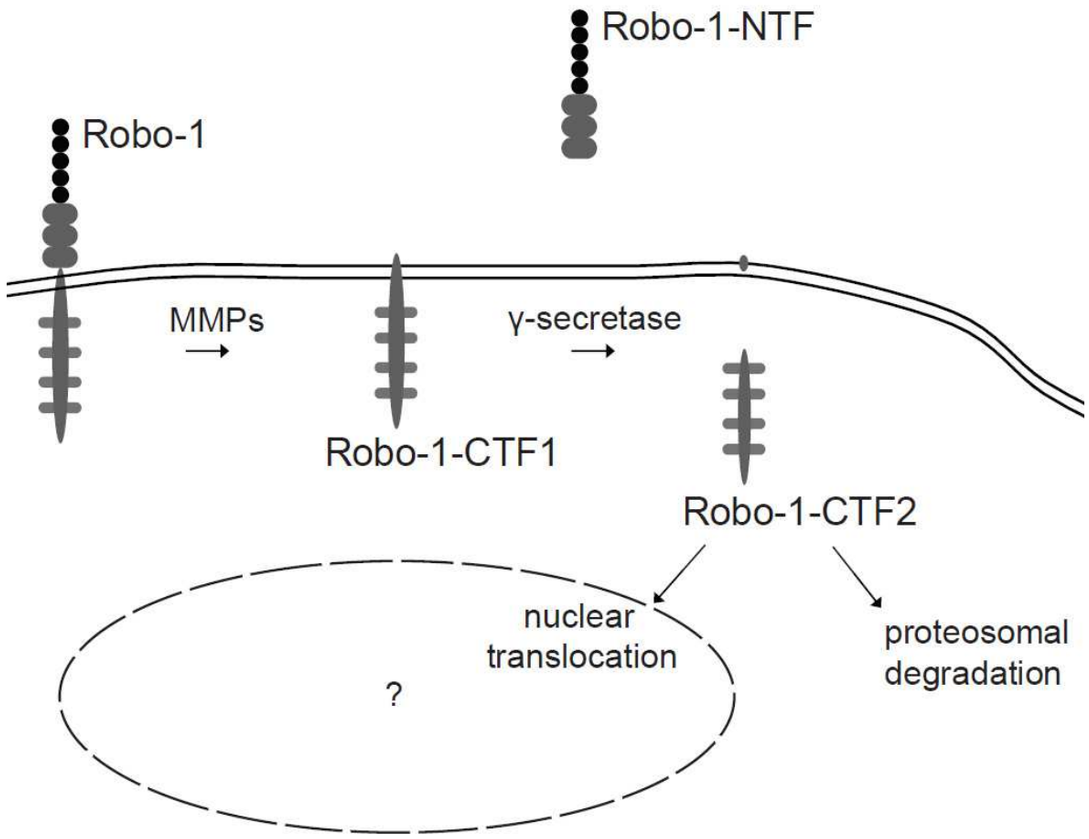

Fig. 4. Successive cleavage of the Robo-1 receptor. Full-length Robo-1 is first cleaved by MMPs to form Robo-1-NTF and Robo-1-CTF1. The second cleavage, mediated by γ-secretase, releases Robo-1-CTF2 which translocates to the nucleus. The function of Robo-1-CTF2 at this location is unknown.

2.2 Slit and robo: Expression

Expression of the Slit genes has been demonstrated in many organisms, including Drosophila (Battye et al., 1999), *Caenorhabditis elegans* (Hao et al., 2001), Xenopus (Chen et al., 2000), *Gallus gallus domesticus* (Holmes et al., 2001; Vargesson et al., 2001), mice (Holmes et al., 1998; Piper et al., 2000), rats (Marillat et al., 2002) and humans (Itoh et al., 1998). In mammals there are three members of the Slit family. Although Slit1 is predominantly expressed in the developing CNS (Yuan et al., 1999), Slit2 and Slit3 are expressed outside the CNS, particularly in lung, kidney, and heart (Wu et al., 2001). Importantly, Slit expression persists in the adult organism, suggesting a role for Slit proteins beyond embryogenesis.

Expression of Robo has been demonstrated in Drosophila (Kidd et al., 1998), mice (Yuan et al., 1999) and humans (Kidd et al., 1998). There are four isoforms of Robo in mammals. Robo-1 is most highly expressed in tissues outside the CNS, including human leukocytes (Wu et al., 2001). Robo-2 is expressed during vertebrate limb development (Vargesson et al., 2001). Robo-3 is expressed following cerebellar and spinal cord lesions (Wehrle et al., 2005). Robo-4 is expressed in the adult organism by primary human endothelial cells, including

umbilical vein endothelial cells and microvascular endothelial cells (Suchting et al., 2005). Interestingly, the tissue expression of Slit and Robo is relatively complementary, suggesting a synergistic relationship (Yuan et al., 1999).

2.3 Slit and robo: Function

Recent studies demonstrate a role for Slit and Robo as repellents outside the CNS. For example, in mesoderm migration in Drosophila, myocyte precursors migrate away from the midline towards peripheral target sites where they fuse to form muscle fibers. In Slit and Robo mutants, these cells do not migrate away from the midline and instead fuse across it (Rothberg et al., 1990). Interestingly, this defect can be reversed by expressing Slit protein in midline cells (Kramer et al., 2001). Slit and Robo signaling also plays a role in nephrogenesis. During renal development, formation of a ureteric bud requires secretion of glial cell derived neurotrophic factor (GDNF) by nearby mesenchymal cells. Slit2 and Robo-2 knockout mice display abnormal patterns of GDNF secretion and develop multiple ureteric buds and multiple urinary collecting systems (Ray, L., 2004). Furthermore, polymorphisms in the human *Robo2* gene are associated with familial vesicoureteral reflux (Bertoli-Avella et al., 2008), a condition involving improper insertion of ureters into the bladder resulting in retrograde flow of urine from the bladder to the kidney. Slit2 also acts as a repellent in the mature organism. A recent study demonstrated that Slit2 inhibits vascular smooth muscle cell migration toward a gradient of platelet-derived growth factor (PDGF) (Liu et al., 2006). This inhibition occurred by suppression of activation of the small GTPase, Rac1. Slit2 has been shown to prevent cancer cell metastasis. The chemokine receptor, CXCR4, is expressed by some human breast cancer cells, allowing them to migrate towards gradients of the CXCR4 ligand, stromal cell-derived factor-1 (SDF-1α), and promoting their metastasis to the lung. Slit2 inhibited chemotaxis, adhesion and chemoinvasion of these breast cancer cells (Prasad et al., 2004). Several other studies have demonstrated a role for Slit2 as a tumor suppressor. Slit2 was shown to inhibit colony formation in lung, colorectal and breast cancer cell lines (Dallol et al., 2002). *Slit2* has also been shown to be epigenetically silenced in more aggressive forms of these and other cancers (Dallol et al., 2003; Dallol et al., 2003; Dickinson et al., 2004). Collectively, these studies demonstrate a repellent role for Slit and Robo in the adult organism and in cancer biology.

The role of Robo-4 signaling in endothelial cells is controversial. Kaur et al. (2006) showed that Robo-4 signaling mediates attractive guidance mechanisms by activating Cdc42 and Rac1 in endothelial cells and inducing actin-mediated cell protrusions, including filopodia and lamellipodia. In fact, Robo-4-induced phenotypic effects in endothelial cells are rescued by dominant negative constructs of Cdc42. Thus, Robo-4 may mediate attractive signaling via activation of Rho-family GTPases, Cdc42 and Rac1. However, in 2008, Jones et al. showed that Slit inhibits endothelial cell migration and angiogenesis. In fact, Robo-4 signaling was shown to stabilize endothelial cell barriers (Jones et al., 2009). Thus, the precise role of Slit/Robo signaling in endothelial cells is yet to be determined.

2.4 Slit2/Robo-1 intracellular signal transduction

Studies of neuronal tissue have demonstrated that Robo-1 signals through two pathways that lead to remodeling of the cytoskeleton: Enabled (Ena) protein and Rho GTPases. Both of

these pathways require the CC motifs in the cytoplasmic domain of Robo. Ena and its mammalian homologue (Mena) are members of a family of proteins that link signal transduction to localized remodeling of the actin cytoskeleton by binding to profilin, an actin binding protein which regulates actin polymerization (Lanier et al., 1999; Wills et al., 1999). Ena is a substrate for Abelson kinase (Gertler et al., 1989). Ena and Abelson both bind to Robo. Ena binds to the CC1 motifs while Abelson binds to the CC3 motif (Fig. 5) (Bashaw et al., 2000). Impairing Ena binding reduces Robo function, while mutations in Abelson result in Robo hyperactivity (Bashaw et al., 2000).

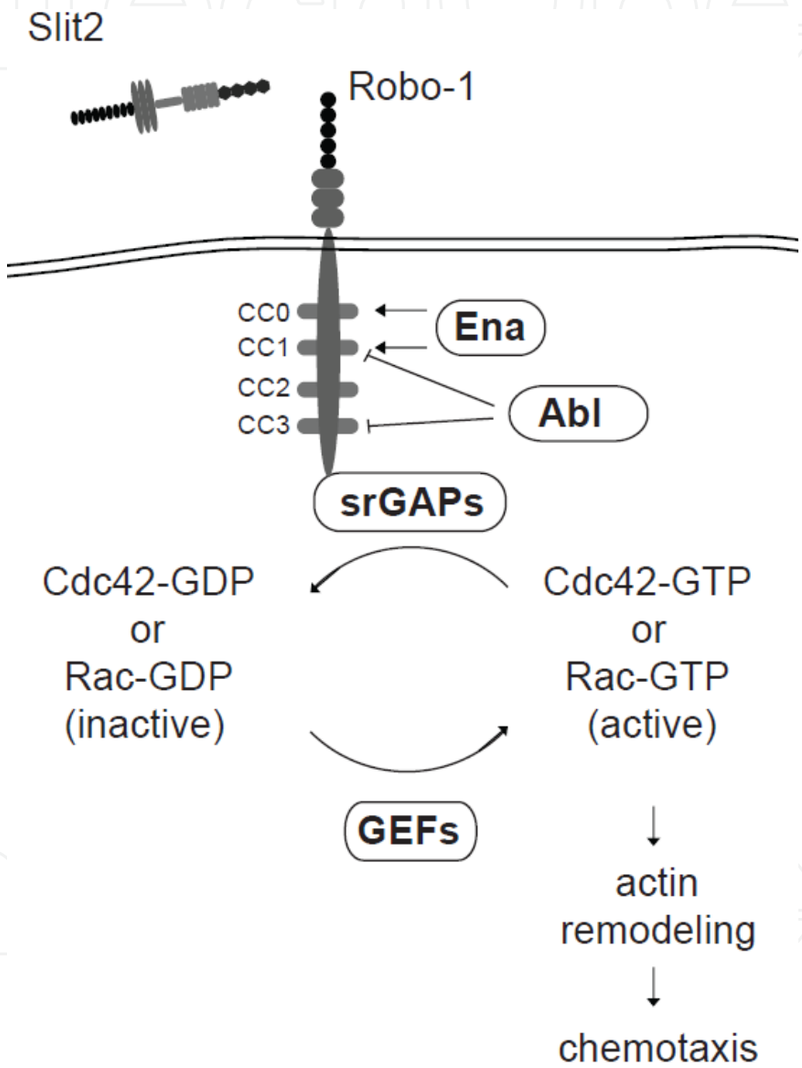

Fig. 5. Intracellular signaling downstream of the Robo-1 receptor. Enabled protein bind to Robo-1 and may contribute to Slit-mediated repulsion. Abelson kinase phosphorylates intracellular domains of Robo and antagonizes Robo function. Ligation of Robo-1 by Slit2 results in the recruitment of srGAPs to the plasma membrane. srGAPs convert active GTP-bound forms of Cdc42 and Rac to their inactive, GDP-bound counterparts, thereby inhibiting the dynamic actin polymerization required for chemotaxis and preventing cell migration.

Slit/Robo also mediate cell repulsion through modulation of Rho GTPase activity. A family of GTPase activating proteins, Slit Robo GTPase activating proteins (srGAPs), were shown to bind Robo (Fig. 5) (Wong et al., 2001). The SH3 domain of srGAP binds the CC3 motif of Robo, while the GAP domain has activity for the Rho GTPases, Rac, Cdc42 and Rho (Wong et al., 2001). Ligation of Robo by Slit induces the recruitment of srGAP, thereby inactivating Rho-family GTPases and inhibiting actin remodeling and cell motility (Wong et al., 2001).

2.5 Slit/Robo in cell trafficking

Both neuronal and leukocyte chemotaxis require recognition of guidance cues, polarization of the cell, and mobilization of the actin cytoskeleton. In addition to repelling developing axons, Slit2 also inhibits chemotaxis of other cell types including vascular smooth muscle cells (Liu et al., 2006). However, the first study to demonstrate that Slit2 inhibits leukocyte chemotaxis, in 2001, utilized transwell migration assays to show that Slit2 inhibits chemotaxis of rat lymph node cells and neutrophil-like HL-60 cells towards MCP-1 and fMLP respectively (Wu et al., 2001). Subsequently, Kanellis et al. (2004) demonstrated that Slit2 inhibits chemotaxis of rat-derived macrophages towards MCP-1 and fMLP. Another study showed that Slit2 inhibited migration of dendritic cells (DCs) (Guan et al., 2003). In 2007, Prasad et al. demonstrated that Slit2 inhibits chemotaxis and transendothelial migration of primary CD4+ T lymphocytes toward SDF-1. Recently, Slit2 was shown to promote chemotaxis of eosinophils towards the chemokine, eotaxin, and to exacerbate allergic airway inflammation (Ye et al., 2010). Thus, Slit2 can negatively or positively regulate directional migration of individual leukocyte subsets.

3. Slit2/Robo-1 signaling inhibits neutrophil migration

Using immunoblotting, we previously demonstrated Robo-1 protein in human and mouse neutrophils (Tole et al., 2009). Immunofluorescence microscopy and flow cytometry revealed that Robo-1 was on the surface of cells.

We used Transwell migration assays to study the effects of Slit2 on chemotaxis of primary human neutrophils. In the presence of Slit2, fMLP-induced migration of neutrophils was inhibited in a dose-dependent manner. In fact, we observed that Slit2 is a potent inhibitor of neutrophil migration toward diverse types of chemotactic cues, including IL-8 and C5a (Tole et al., 2009).

Neutrophil exposure to chemoattractants results in the activation of the Rho GTPases, Rac and Cdc42 and the subsequent reorganization of actin filaments. (Sun et al., 2004; Srinivasan et al., 2003). Since the predominant isoform of Rac in human neutrophils is Rac2, not Rac1, the activation of Rac2 was studied. Following stimulation with fMLP, levels of activated Cdc42 and Rac2 in the presence of Slit2 were less than half of those observed in untreated control cells.. We found that Slit2 inhibits neutrophil chemotaxis and actin polymerization by preventing cell polarization and disrupting generation and recruitment of activated Rac2 and Cdc42.

We examined the effects of Slit2 on the activation of kinase signaling pathways associated with neutrophil chemotaxis, namely, PI3K, Akt, Erk, and p38 MAPK. Stimulation of neutrophils with fMLP resulted in levels of activated Akt that were comparable in the presence or absence of Slit2, indicating that Slit2 does not impair the ability of neutrophils to

generate membrane PIP_3. Similarly, Slit2 treatment had no effect on fMLP-induced phosphorylation of Erk and p38 MAPK. Thus, Slit2 inhibits neutrophil chemotaxis by specifically preventing activation of Cdc42 and Rac2 but not activation of Akt, Erk, or p38 MAPK (Tole et al., 2009).

We studied the effect of Slit2 on neutrophil recruitment *in vivo* using a mouse model of chemical irritant peritonitis (Glogauer et al., 2003). The administration of Slit2 prior to induction of peritonitis with sodium periodate, resulted in a significant decrease in neutrophil recruitment to the peritioneum.. Slit2 also prevented neutrophil recruitment to the peritoneal cavity in response to other chemoattractant factors tested, including C5a and MIP-2. These data demonstrate that Slit2 acts as a potent inhibitor of chemotaxis for circulating neutrophils toward diverse inflammatory stimuli. Slit2 also inhibited infiltration of other leukocyte subsets, especially monocytes/macrophages (Tole et al., 2009).

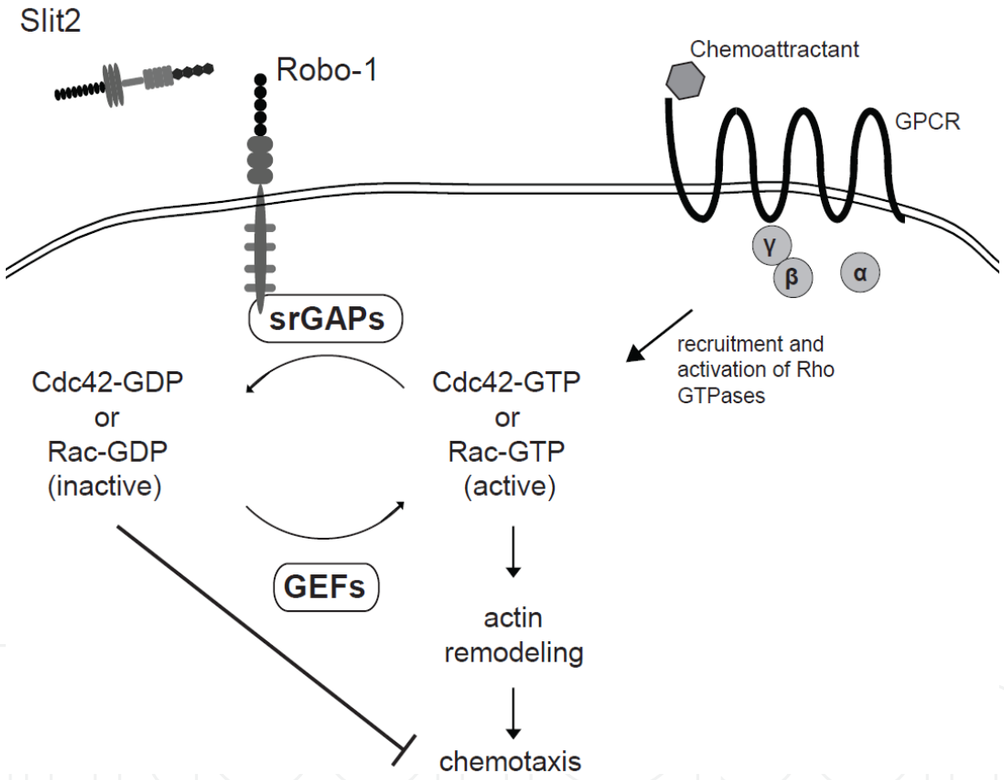

Fig. 6. Slit2/Robo-1 signaling inhibits actin remodelling required for chemotaxis. Chemoattractant signaling induces the activation of Rho GTPases Cdc42 and Rac, allowing for actin remodeling and chemotaxis. The binding of the LRRs on Slit2 to Robo-1 recruits srGAPs to the membrane, converting active Rho GTPases to their inactive, GDP-bound, forms. Inactivation of Rho GTPases abolishes actin remodeling and prevents cell chemotaxis.

4. Conclusion and discussion

The LRRs contained in Slit proteins can inhibit the migration of diverse cells, including neuronal cells and vascular smooth muscle cells. The conserved structure of Slit proteins

also allows them to inhibit the migration of several subsets of leukocytes, including DCs and lymphocytes (Guan et al., 2003; Kanellis et al., 2004; Prasad et al., 2007). We have recently shown that Slit2 inhibits migration of neutrophils to diverse inflammatory attractants, *in vitro* and *in vivo*. Furthermore, we have demonstrated that this inhibition is mediated by inactivation of Rho-family GTPases, Rac and Cdc42 (Fig. 5). Excessive infiltration of leukocytes, particularly neutrophils, is associated with local tissue damage seen in inflammatory conditions such as rheumatoid arthritis and ischemia reperfusion injury (Weissmann et al., 1984; Kaminski et al., 2002). Thus, the protein structure of the conserved LRR regions contained in Slit proteins may be utilized as a novel therapeutic strategy to locally inhibit leukocyte recruitment.

Extensive glycosylation makes Slit2 a large and relatively "sticky" protein, potentially allowing it to maintain a high local concentration through adherence to extracellular matrix proteins such as glypican-1 (Ronca et al., 2001). Thus, after regional administration, Slit2 may be retained at sites of inflammation, such as joints and transplanted organs, thereby alleviating neutrophil-inflicted tissue injury associated with rheumatoid arthritis and ischemia reperfusion injury. As Slit2 blocks migration of several types of inflammatory cells, including neutrophils, T lymphocytes, macrophages, and dendritic cells, toward diverse chemoattractant signals, it could act as a highly effective anti-inflammatory agent (Guan et al., 2003; Kanellis et al., 2004; Prasad et al., 2007; Wu et al., 2001). Further studies are required to explore the therapeutic use of Slit2, or of a Slit-like compound containing the structurally critical LRRs, for prevention and treatment of localized inflammation.

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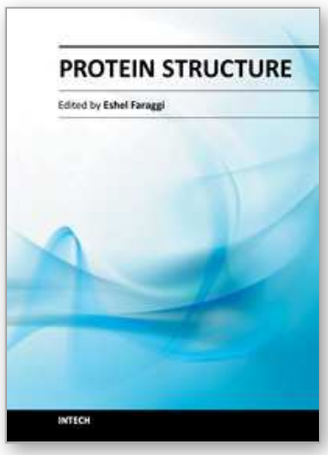

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Since the dawn of recorded history, and probably even before, men and women have been grasping at the mechanisms by which they themselves exist. Only relatively recently, did this grasp yield anything of substance, and only within the last several decades did the proteins play a pivotal role in this existence. In this expose on the topic of protein structure some of the current issues in this scientific field are discussed. The aim is that a non-expert can gain some appreciation for the intricacies involved, and in the current state of affairs. The expert meanwhile, we hope, can gain a deeper understanding of the topic.

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