STRUCTURAL BASIS OF MEMBRANE TARGETING OF THE INNATE IMMUNITY ADAPTOR TIRAP BY ITS PHOSPHOINOSITIDE-BINDING MOTIF

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences

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June 17, 2016
Blacksburg, Virginia

Keywords: TLR, TIRAP, NMR, phosphoinositide

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Toll-like receptors (TLRs) are the main components of the innate immunity. Pathogen-activated TLRs trigger a cytoplasmic signaling cascade through adaptor proteins, with the first being the TIR domain-containing adaptor protein (TIRAP). TIRAP contains a TIR domain, which associates with TLRs and other adaptor proteins; and a N-terminal phosphoinositide-binding motif (PBM) that mediates the membrane recruitment of TIRAP. Upon ligand activation, TLRs are recruited to the phosphoinositide (PIP)-enriched region in the membrane, where TIRAP recruits other adaptors to the membrane to activate TLR signaling pathway. To investigate the mechanism of membrane targeting of TIRAP and the basis for its regulation, I functionally and structurally characterized TIRAP and its PBM using biophysical approaches. I show that TIRAP PBM adopts helical structural in dodecylphosphocholine (DPC) micelles and other membrane mimics. NMR studies reveal that TIRAP PBM binds PIPs following a fast exchange regime with a moderate affinity through two conserved basic termini. Mutation of these two basic regions abolishes PIPs binding without distorting the helical structure of the peptide. Solution NMR structure of TIRAP PBM exhibits a central relatively hydrophobic helix surrounded by the flexible N- and C-termini. Paramagnetic studies indicate that the helix is close to the micelle core, whereas two termini are located on the micellar surface. Nuclear spin relaxation experiments indicate that the two termini of TIRAP PBM become more ordered when
bound to PIP, thus, we propose that the central helix in PBM is responsible for membrane insertion, whereas the two sets of basic residues interact with PIPs to stabilize TIRAP’s membrane interaction. Phosphomimetic mutation of Thr28 to Asp (T28D) as well as phosphorylation in Thr28 inhibit TIRAP PBM’s binding to phosphoinositides by distorting the central helical structure of the peptide. More importantly, TIRAP T28D disrupts its subcellular localization in vivo. Thus, phosphorylation can impair proper insertion of TIRAP at the plasma membrane through PBM and, consequently, it may represent the first signal that promotes TIRAP degradation.
DEDICATION

This thesis is dedicated to:

My beloved husband Yu Fu

My dear parents Fu-Tang Zhao & Ai-Ying Yang
ACKNOWLEDGMENTS

This thesis recorded my five-year long journey on the road of scientific research. It would not have been happened without the help of so many people in so many ways. I am grateful to many of them for my achievement today.

My deepest gratitude goes to my advisor, Dr. Daniel G.S. Capelluto, who expertly guided me through my graduate education and shared the excitement of many discoveries on my research. I am thankful to him for his encouragement and patience, which are so much important in scientific research, especially when things do not work. He can always find positive side, and help to move research to next stage.

My appreciation goes to all my committee members, Dr. Tijana Grove, Dr. Peter Kennelley, Dr. Liwu Li (alphabetically) for their great idea and suggestion on this project. They have been so helpful on solving problems and providing new direction on my research. Many thanks go to Dr. Carla Finkielstein who has been very generous to help with cellular studies reported in this thesis. All the in vivo experiments are conducted in her cell culture room. Many thanks go to Drs. Geoffrey Armstrong and Jeffrey Ellena for their help on NMR studies. I also want to thank all Capelluto-Finkielstein lab members, for their guidance, especially Dr. Shuyan Xiao, who taught me everything about NMR, Dr. Tetsuya Gotoh for his help on cell culture, Mary Kate Brannon, Sam Berk, Ayana Stukes, and Taylor Rose.

I would like to give my heartiest thanks to some of the family members and friends I’ve met in Blacksburg. First and foremost, my sincere love to my husband Yu Fu, who has
been stand beside me throughout these years. I want to thank my parents for their understanding and support, and I am sorry for being so far away. I want to thank my sisters, brother, my grand parents, and my aunts who always bring me joy and love. My gratitude goes to all of my friends in Blacksburg, who have accompanied me in my hardest time, who have supported and listened to me. Among others, Wenmeng Tian, JingJing Liu, Xiangping Fu, Shengchen Su, Hui Deng, Xu Feng, Hui Yi, Ting Guan, Lin Kang, and Xin He are great people who have made my graduate life in Virginia Tech a wonderful one!
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<th>Description</th>
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<tbody>
<tr>
<td>Aβ</td>
<td>Fibrillar amyloid β</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>BCAP</td>
<td>B-cell adaptor for PI3K</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>CLRs</td>
<td>C-type lectin receptors</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic-AMP response element binding protein</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Damage-associated molecular patterns</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DD</td>
<td>Death domain</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s minimal essential medium</td>
</tr>
<tr>
<td>DPC</td>
<td>Dodecylphosphocholine</td>
</tr>
<tr>
<td>DSA</td>
<td>Doxyl steric acid</td>
</tr>
<tr>
<td>DTT</td>
<td>D-L-dithiothreitol</td>
</tr>
<tr>
<td>ECD</td>
<td>Ectodomain</td>
</tr>
<tr>
<td>ENTH</td>
<td>Epsin N-terminal homology</td>
</tr>
<tr>
<td>FYVE</td>
<td>Fab1, YOTB/ZK632, Vac1p, and EEA1</td>
</tr>
<tr>
<td>GLUE</td>
<td>GRAM-Like Ubiquitin-binding in EAP45</td>
</tr>
<tr>
<td>Gp96</td>
<td>Glycoprotein 96</td>
</tr>
<tr>
<td>GRAM</td>
<td>Glucosyltransferase, Rab-like GTPase activators and</td>
</tr>
</tbody>
</table>
Myotubularins domain

HBV  Hepatitis B virus
HSP  Heat-shock protein
IFN  Interferon
IFNGR  Interferon-gamma (IFN-γ) receptor
IKK  Inhibitor of kappa-β-kinase
IL-1R  Interleukin-1 receptor
IP₃  Inositol (1,4,5)-trisphosphate
IP10  IFN-inducible protein 10
IPTG  Isopropyl-β-D-thiogalactopyranoside
IRAK  Interleukin-1 receptor-associated kinase
JNK  c-Jun N-terminal kinase
LPS  Lipopolysaccharide
LTA  Lipoteichoic acid
LRRs  Leucine-rich repeats
MAPKs  Mitogen activated protein kinase
MKK  MAPK kinase
MyD88  Myeloid differentiation primary protein 88
NEMO  NF-κB essential modulator
NK  Natural killer
NLRs  Nucleotide-binding domain and leucine-rich repeat containing receptors
NMR  Nuclear Magnetic Resonance
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOE</td>
<td>Nuclear Overhauser effect</td>
</tr>
<tr>
<td>NSR</td>
<td>Nuclear spin relaxation</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBD</td>
<td>N-terminal phosphoinositide-binding domain</td>
</tr>
<tr>
<td>PBM</td>
<td>N-terminal phosphoinositide-binding motif</td>
</tr>
<tr>
<td>PEST</td>
<td>proline, glutamic acid, serine and threonine</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PHD</td>
<td>Plant homeodomain</td>
</tr>
<tr>
<td>PLA2</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLOA</td>
<td>Protein-lipid overlay assay</td>
</tr>
<tr>
<td>PML</td>
<td>Peripheral mononuclear leukocytes</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine-binding domain</td>
</tr>
<tr>
<td>PtdIns(3)P</td>
<td>Phosphatidylinositol-3-phosphate</td>
</tr>
<tr>
<td>PtdIns(4,5)P_2</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PX</td>
<td>Phox homology</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid inducible gene I</td>
</tr>
<tr>
<td>RLRs</td>
<td>RIG-I-like receptors</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>SARM</td>
<td>Sterile α- and armadillo-motif-containing protein</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOCS-1</td>
<td>Suppressor of cytokine signaling-1</td>
</tr>
<tr>
<td>TAB1/2</td>
<td>TAK1 binding protein 1/2</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming growth factor β activating kinase 1</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TBK1</td>
<td>TRIF associated NF-κB activator binding kinase 1</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll-interleukin-1 receptor</td>
</tr>
<tr>
<td>TIRAP</td>
<td>TIR domain-containing adaptor protein</td>
</tr>
<tr>
<td>TIRAP PBM-P</td>
<td>Thr28 phosphorylated in TIRAP PBM</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TRAF-6</td>
<td>Tumor necrosis factor receptor-associated factor 6</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR domain containing adaptor protein inducing interferon-β</td>
</tr>
</tbody>
</table>
CHAPTER I: LITERATURE REVIEW

This thesis focuses on the molecular basis of membrane targeting of the innate immunity adaptor, the Toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP). TIRAP interacts with the activated Toll-like receptor (TLR), triggering innate immunity signaling. The following literature review outlines the pathways in which TIRAP plays a key role and provides the background information on TIRAP’s function in innate immunity.

PART I INNATE IMMUNITY AND TLR SIGNALING PATHWAY

1.1 Innate Immunity and PRRs

In vertebrates, the immune system is typically divided into two categories: innate and adaptive immune systems. Adaptive immunity refers to antigen-specific, and generating receptors requires rearrangement of receptor gene segments. Adaptive immunity is able to distinguish closely related pathogenic molecules as well as remember and responds to the repeated exposure of the same molecule. Innate immunity is pathogen-specific, relies on a number of germline-encode receptors, known as pattern recognition receptors (PRRs), and it is the immediate first line of defense mechanism found in all multicellular organisms (Table 1) [1].

Innate immune system provides a broad host defense against pathogenic microbes and forms the foundation of immune system. Innate immune cells, including macrophages,
dendritic cells (DCs), mast cells, and neutrophils, among others, become activated during an inflammatory response due to infection. These cells rapidly differentiate into short-lived effector cells to remove invading pathogens. If successful, the adaptive immunity will not be activated, otherwise, they will express co-stimulatory molecules, such as CD80 and CD86, to trigger adaptive immunity [1].

Table 1. Innate and adaptive immunity

<table>
<thead>
<tr>
<th>Property</th>
<th>Innate Immune System</th>
<th>Adaptive Immune System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptors</td>
<td>Fixed in genome, Rearrangement is not necessary</td>
<td>Encoded in gene segments, Rearrangement necessary</td>
</tr>
<tr>
<td>Recognition</td>
<td>Conserved PAMP and DAMP</td>
<td>Details of molecular structure</td>
</tr>
<tr>
<td>Action time</td>
<td>Immediate activation of effectors</td>
<td>Delayed activation of effectors</td>
</tr>
<tr>
<td>Effectors</td>
<td>Cytokine (IL-1β, IL-6), Chemokine (IL-8), Co-stimulatory molecules</td>
<td>Effect or cytokines: (IL-4, IFNγ), IL-2</td>
</tr>
</tbody>
</table>

Innate immune cells recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) by their multiple, distinct PRRs [2], which can be divided into secreted, transmembrane, and cytosolic classes [3], and lead to pro-inflammatory signaling pathways. PRRs are universally expressed in professional and non-professional cells. Secreted PRRs, including collectins, ficolins and pentraxins, bind to microbial cell surface to opsonize pathogens for phagocytosis by macrophages and neutrophils. The cytosolic PRRs, including the retinoic acid inducible gene I (RIG-I) like receptors (RLRs) and the nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs). RLRs detect viral pathogens by recognizing their RNAs and DNAs [4] whereas NLRs detect pathogens and stress signals, such as degradation
products of peptidoglycans, microbial products and non-infectious crystal particle [5]. The transmembrane PRRs include the TLRs and the C-type lectin receptors (CLRs). CLRs, such as Dectin-1 and Dectin-2 recognize β-glucans on fungal cell walls [6]. In mammals, TLRs are expressed on both plasma and endosomal/lysosomal membranes. Plasma membrane TLRs detect conserved PAMPs, such as lipopolysaccharide (LPS) of gram-negative bacteria, lipteichoic acid (LCA) of gram-positive bacteria and bacteria lipoproteins and flagellin, whereas endosomal TLRs recognize microbial nucleic acids, such as dsRNA, ssRNA, and dsDNA. Most PRRs initiate common downstream signaling pathway by activating transcription factors, such as NF-κB [6].

1.2 The TLRs

1.2.1 Introduction to TLRs

TLRs are the best-characterized PRRs that are ubiquitously expressed in a variety of immune cells, including macrophages, DCs, neutrophils, eosinophils, T cells, B cells, mast cells, and epithelial cells (Table 2) [7]. The Toll protein, which plays a pivotal role in antifungal defense, was first identified in Drosophila melanogaster [8]. One year later, the human homologue of Toll protein (TLR4) was reported by Medzhitov and colleagues [9], termed Toll-like receptor. To date, there are thirteen members of TLR family that have been identified and characterized in mammals. TLR1 to TLR9 are conserved in both humans and mice. TLR10 is expressed in humans only, whereas TLR11 to TLR13 are present in mice. Different TLRs have specific intracellular location. TLR1, TLR2, TLR4, TLR5, and TLR6 are localized on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 are expressed at the surface of intracellular vesicles [10]. Each TLR is able to
recognize a particular molecular pattern. For example, TLR2, 4, 5, 6 and 11 bind to bacterial membrane associated molecules such as LPS, lipoproteins, and peptidoglycan, whereas TLR3, 7, 8 and 9 detect viral and bacterial or endogenous DAMPs, such as ssRNA (Table 2).

Table 2. Toll-like receptors and their expression pattern on immune cells, ligands, and cytokine production

<table>
<thead>
<tr>
<th>TLR</th>
<th>Immune cells</th>
<th>Cellular location</th>
<th>Exogenous ligands</th>
<th>Endogenous ligands</th>
<th>Signal adaptor</th>
<th>Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR 1</td>
<td>Most cell types including DCs and B cells</td>
<td>Cell surface</td>
<td>Bacteria, triacyl-lipopeptides</td>
<td>Unknown</td>
<td>MyD88</td>
<td>Cytokines</td>
</tr>
<tr>
<td>TLR 2</td>
<td>PML, DCs, monocytes and T cells</td>
<td>Cell surface</td>
<td>Bacteria, peptidoglycan, lipoproteins, LTA fungi: zymosan</td>
<td>HSP60, HSP70, Gp96</td>
<td>MyD88/TIRAP</td>
<td>Cytokines</td>
</tr>
<tr>
<td>TLR 3</td>
<td>DCs, NK cells and T cells</td>
<td>Endosome</td>
<td>Viruses: dsRNA</td>
<td>mRNA</td>
<td>TRIF</td>
<td>Cytokines, type I IFNs</td>
</tr>
<tr>
<td>TLR 4</td>
<td>Macrophages, DCs and T cells</td>
<td>Cell surface</td>
<td>Bacteria: LPS, Viruses: RSV fusion protein, Fungi: mannan Protozoa: Glycoinosito</td>
<td>HSP22, 60, 70, 72, Gp96, HMGB1, fibronectin, oxidized phospholipid</td>
<td>MyD88/TIRAP/TRAM/TRIF</td>
<td>Cytokines, type I IFNs</td>
</tr>
<tr>
<td>TLR 5</td>
<td>Monocytes, DCs, NK cells and T cells</td>
<td>Cell surface</td>
<td>Bacteria: flagellin</td>
<td>Unknown</td>
<td>MyD88</td>
<td>Cytokines</td>
</tr>
<tr>
<td>TLR 6</td>
<td>B cells, DCs, monocyte and NK cells</td>
<td>Cell surface</td>
<td>Diacyl lipopeptides, LCA zymosan</td>
<td>Unknown</td>
<td>MyD88</td>
<td>Cytokines</td>
</tr>
<tr>
<td>TLR 7</td>
<td>B cells, DCs, monocyte and T cells</td>
<td>Endosomal compartment</td>
<td>Viruses: ssRNA</td>
<td>ssRNA (immune complex)</td>
<td>MyD88</td>
<td>Cytokines, type I IFNs</td>
</tr>
<tr>
<td>TLR 8</td>
<td>Monocytes, DCs, NK and T cells</td>
<td>Endosomal compartment</td>
<td>Viruses: ssRNA</td>
<td>ssRNA (immune complex)</td>
<td>MyD88</td>
<td>Cytokines, type I IFNs</td>
</tr>
</tbody>
</table>
1.2.2 The representative TLR structure

TLRs are type I integral membrane glycoproteins, whose molecular weights range from 90-115 kDa, each with an N-terminal ligand recognition ectodomain (ECD), a single transmembrane helix, and a C-terminal globular cytoplasmic signaling domain [11]. The transmembrane domains of TLRs typically contain a stretch of approximately 20 uncharged, mostly hydrophobic residues [12]. The ECDs of TLR are glycoproteins that either face the exterior of the cell or the lumen of an intracellular compartment, such as endosome or lysosome, where they encounter and recognize molecule released by invading pathogens [11]. The ECD structure is composed of an N-terminal cap, a leucine-rich repeats (LRRs) domain, and a cysteine-rich domain. The LRR domain contains 19-25 leucine-repeats appearing in an xLxxLxLxx motif within a stretch of 550-800 residues. In ECDs, each individual LRR forms a loop, and multiple consecutive LRRs from a curved solenoid, in which the consensus hydrophobic residues point inward and form the core of the solenoid. The beginning of LRR motif that presents in all LRR subtypes form a β-strand, whereas the remaining portion is variable. This portion forms an alpha helix aligned with other helix, and build the convex surface of the solenoid. As a result, each LRR protein contains a concave core, a convex surface, and an ascending lateral loop that connects the beta strand in the core to the helical convex
surface. Most LRR proteins bind to ligands through their concave surface, which is highly diverse in amino acid sequence. These structural variations of TLR ECDs likely provide the specificity needed to recognize the ever-growing list of PAMPs. The ECD of TLR4, contains 21 LRRs, forms a complex with a co-receptor, MD-2, to recognize LPS from Gram-negative bacteria [13]. MD-2 is anchored by several hydrogen bonds to the lateral and concave surface of TLR4-ECD and contacts residues from the LRR2-LRR10 area.

The cytoplasmic signaling domain of TLRs is called the TIR domain since it is a homologue of the signaling domain of interleukin-1 receptor (IL-1R) family members. Unlike ECDs, which is variable among TLRs, TIR is very conserved. Besides TLRs, adaptor proteins, such as MyD88, TIRAP, TRIF, and TRAM, also contain a C-terminal TIR domain [12]. Upon activation of TLR signaling by ligand-induced dimerization of the ECDs, TLR TIR domains dimerize, and recruit adaptor molecules through TIR-TIR interactions [14]. In all TIR domains, there are five-stranded parallel $\beta$-sheet (\(\beta A-\beta E\)) surrounded by five $\alpha$ helices (\(\alpha A-\alpha E\)), the loop connects $\beta$ strand B and helix B termed BB-loop, which is important for TLR dimerization and adaptor recruitment upon ligand binding [15].

1.3 TLR signaling

1.3.1 Ligand binding of ECD

Ligand binding by the ECD of TLRs leads to dimerization of the intracellular TIR domain, and this event recruits MyD88 and TRIF depending on which TLR is activated. The TLR pathway that employs MyD88 is termed MyD88-dependent pathway, whereas
the pathway that recruits TRIF is called MyD88-independent or TRIF pathway. TLR1/TLR2, TLR2/TLR6, TLR5, TLR7, TLR8, and TLR9 signal through the MyD88-dependent pathway, TLR3 signal through TRIF/MyD88-independent pathway, and TLR4 use both MyD88 and TRIF pathways. Among the TLRs using MyD88, TLR2 and TLR4 do not interact with MyD88 directly. Their association requires TIRAP, which mediate interaction between MyD88 and activated TLRs [14]. The promiscuity of lipid binding by the phosphoinositide (PIP)-binding domain (PBD) of TIRAP allows it to sample multiple compartments, hence, it diversifies the subcellular sites of TLR signal transduction [16]. TLR4, in the plasma membrane, recognizes LPS in a MD-2-dependent fashion; two copies of TLR4-MD2-LPS interact symmetrically to form a TLR4 homodimer [17], then sequentially recruit TIRAP and MyD88 to pass signal to downstream effectors. Then, TLR4 translocate to the endosome through a dynein-dependent mechanism to induce a TRIF-dependent pathway [18].

1.3.2 MyD88-dependent pathways

MyD88 contains a TIR domain, which interact with TIR of TLRs and TIRAP, and a death domain (DD). In MyD88-dependent pathways, after ligand binding at the plasma membrane (TLR2/TLR1, TLR2/TLR6, TLR4/MD-2 and TLR5) or endosomal membrane (TLR7, TLR8 and TLR9), TLRs recruit MyD88 or TIRAP/MyD88 to their TIR domains, and the DD of MyD88 recruits the interleukin-1 receptor-associated kinase (IRAK) proteins [14]. IRAK proteins consist of an N-terminal DD and a central serine/threonine-kinase domain. IRAK4 is the first IRAK sequestered by TLR-MyD88-TIRAP complex. Subsequently, the Tollip-IRAK-1 (or IRAK-2) complex, which is in the cytosol at steady
state condition is also recruited by DD-DD interactions with IRAK4 [14]. IRAK4 phosphorylates IRAK1 on key threonine residues, and in turn, promotes the auto phosphorylating activities of IRAK1 [19]. IRAK1 undergoes degradation within 1 h of activation, and then IRAK2 functions to maintain sustained TLR signaling [20]. The MyD88-IRAK1/2-IRAK4 complex, so-called myddosome [21], then recruits TRAF-6, by associating with phosphorylated IRAK1. Phosphorylated IRAK1 and TRAF6 then dissociate from the receptor and form a complex with the transforming growth factor β activating kinase 1 (TAK1), TAK1 binding protein 1 (TAB1) and TAB2, which induces the phosphorylation of both TAK1 and TAB2. Following TAK1 and TAB1 phosphorylation, IRAK is degraded at the plasma membrane, and the remaining complex translocate to the cytosol to further phosphorylate IkB kinase (IKK) and MAP kinase (MAPKs). IKK is composed of two kinase subunits, IKKα and IKKβ, and a regulatory subunit so-called IKKγ or NF-κB essential modulator (NEMO). IKK complex (IKKα/IKKβ/NEMO) catalyzes the phosphorylation of NF-κB inhibitory protein IkBα, which undergoes proteasome degradation to release NF-κB to translocate into the nucleus and induces pro-inflammatory gene expression [22]. TAK1 also phosphorylates MKKs to induce another two downstream pathway to activate transcription factors. TAK1 phosphorylates the mitogen-activated protein kinase kinase (MKKs), and the activated MKK3 phosphorylates the stress activated protein kinase p38, leading to cAMP response element binding (CREB) nuclear transcription factor activation, whereas phosphorylation of MKK4/7 leads to Jun N-terminal kinase (JNK) mediated activation of activator protein-1 (AP-1) transcription factor [23]. The NF-κB and AP-1
pathways together regulate the expression of pro-inflammatory cytokines, chemokines, and MHC co-stimulatory molecules [22].

1.3.3 MyD88-independent pathway/TRIF pathway

After activation of TLR3/4, another adaptor protein, TIR-domain containing adaptor protein inducing interferon-β (TRIF), is recruited to TIR domain of TLRs through TIR-TIR interactions. TLR3 only uses TRIF as its adaptor molecules, whereas TLR4 does not directly interact with TRIF, and instead, it requires another sorting adaptor, the TRIF-related adaptor molecule (TRAM). Like TIRAP, TRAM bridges TRIF to TLR4. TLR4 is the only TLR, which signals through both MyD88-dependent and TRIF-dependent pathways. The sorting adaptors TIRAP and TRIF play important role in determining which pathway TLR4 activates. TRIF recruits the tumor-necrosis factor receptor associated factor 3 (TRAF3) and the TRIF associated NF-κB activator binding kinase 1 (TBK1) in order to phosphorylate IRF3 [24]. Phosphorylated IRF3 activates, in conjunction with p300 and CREB binding protein (CBP), the expression of interferon inducible genes, IP-10 and RANTES [24].

1.4 TLR signaling in human diseases and therapeutic treatments

TLR signaling plays a key role in both innate and adaptive responses. It senses the presence of pathogens, provokes immediate anti-pathogen response, and stimulates long-lasting adaptive response. There is no doubt of importance of its role, however, aberrant activation of TLR signaling pathway can be troublesome. Accumulating evidence show that TLR signaling has been associated with several severe human diseases, such as cancer, and cardiovascular, neurodegenerative, and immunity-related
diseases. Thus, the interest of scientists and clinicians in developing immune-stimulatory agents targeting TLRs has been intriguing. Moreover, TLR agonists and antagonists have been approved by regulatory agencies for their use in human subjects including cancer patients.

1.4.1. TLRs in cancer

Increasing evidence points to the connection between TLRs and risk of cancer. Lu et al reported that colorectal cancer patients have elevated expression of TLR1, 2, 4 and 8 mRNA in comparison with normal individuals [25]. Droemann and colleagues demonstrated that TLR9 is expressed in human lung cancer tissues and other tumor cell lines, however, the non-malignant lung tissue showed very weak expression [26]. Another study characterized that TLR9 expression is significantly increased in prostate cancer epithelium and stroma, especially in the most poorly differentiated forms [27]. Other reports also showed that TLR is associated with breast and gastric cancer [28, 29]. There is a clear role of TLRs in cancer, whereas the underlying mechanism of TLRs in the development of these cancers is not fully understood.

1.4.2 TLRs in cardiovascular diseases

It has been reported that TLR signaling pathway has been associated with several cardiovascular diseases including atherosclerosis, ischemic heart disease, heart failure and ischemic reperfusion injury [30-34]. TLR1/TLR2 and TLR4 expression are markedly enhanced in human atherosclerotic plaques [35], which may induce chronic inflammation, and eventually lead to endothelial cell dysfunction, elevated coagulation and increased atherosclerotic progression [36]. Atherosclerosis causes ischemic heart
disease, which is the leading cause of death worldwide. TLR2 and TLR4 deficiency has been reported attenuate myocardial inflammation, reduce infarct size, and preserves ventricular function after transient ischemic injury [37].

1.4.3 TLRs in neurodegeneration diseases

TLRs are found in many central nervous system cells, such as microglial cells astrocytes, and oligodendrocytes [38]. New evidence implicated that these receptors play a critical role in neuronal homeostasis [39]. TLRs have been found to increase the recognition of fibrillar amyloid β (Aβ) by microglial cells through their interaction with other receptors [40]. Another study demonstrated that TLR2 deficiency accelerates spatial and contextual memory impairments that associate with increased level of Aβ, indicating TLR2 plays a positive role in preventing from toxic Aβ [41].

1.4.4 TLRs in autoimmune diseases

The link between TLRs and autoimmune diseases has become apparent. TLRs can be activated not only by exogenous PAMPs, but also endogenous DAMPs, such as heat shock proteins, fibronectin, fibrinogen, unknown factors from injured myocardium and necrotic cells, and exaggerate activation of TLRs may induce autoimmune diseases [22]. Systemic lupus erythematosus (SLE) is one of the classic autoimmune diseases along with others. SLE patients have autoantibodies against self-antigens, including DNA. Normal people also have antibody to DNA, which are specific for bacterial DNA, however, SLE patients have antibodies recognize DNA backbones that are common to DNA of all species, including human. It has been reported that TLR9 might be responsible for recognition of immune complexes containing DNA in SLE patients [42].
1.4.5 Therapeutic treatments targeting TLRs

Since the bulk of the data suggests that TLR pathway plays a key role in human disease pathogenesis, targeting TLRs and downstream protein molecule provide an effective therapeutic approach for indicating disease. Modulation by both stimulation and inhibition of TLR signaling has been explored. TLR agonists have been studied to function as vaccine adjuvants for inflammation-related disease. Two hepatitis B virus (HBV) vaccines that use TLR4 agonists have been approved [43]. Fendrix is a HBV vaccine that targets TLR4, and is manufactured by GlaxoSmithKline, and approved in Europe; Supervax, another HBV vaccine targets TLR4, was approved in Argentina. Bacillus Calmette-Guerin is a well-recognized immunotherapeutic agent used for preventing from tuberculosis. It has been used for the treatment of patients with bladder cancer by stimulating the immune response via activation of TLR4 [44]. There are many other TLRs agonists, such as TLR5, TLR7/TLR8, TLR9 agonists, that are in clinical development [43]. TLR agonists have been investigated for treatment of cancer, and viral infections; while TLR antagonists that inhibit or reduce TLR signaling have been developed for chronic inflammation related diseases and autoimmune diseases. TLR4, TLR7/TLR9 antagonists have been developed target severe sepsis and lupus by binding receptor but fail to signal [43].
PART II: TIRAP IN TLR SIGNALING

2.1 Adaptors in TLR signaling

In order to propagate signal of pathogen to nucleus and initiate pro-inflammatory responses, TLR must recruit adaptor proteins to trigger a signaling cascade. To date, there are 6 well-established cytosolic adaptor molecules including TIRAP, MyD88, TRIF, TRAM, sterile α- and armadillo-motif-containing protein (SARM), and B-cell adaptor for PI3K (BCAP) [45]. As discussed above, MyD88 signal for all TLRs except TLR3; TLR3 uses TRIF to propagate signal to downstream; TLR4 use both MyD88 and TRIF, thus, it needs a sorting adaptor, TIRAP bridges MyD88 to TLR4, whereas TRAM bridges TRIF to TLR4; all these four adaptors are necessary for proper initiation of TLR signaling. Among these adaptor proteins, TIRAP is critical for signaling from plasma membrane by TLR2 and TLR4, which sense a broad spectrum of pathogenic organisms. It was previously excluded as an adaptor for endosomal TLRs, such as TLR9 [24]; however, a recent study showed that TIRAP is necessary for endosomal TLR signaling in response to viral infection [16], which indicates that TIRAP has a widespread role in TLR signaling transduction, but that the mechanism of TIRAP membrane association is currently unknown.

2.2 TIRAP’s structure and binding site for other TLR signaling-related proteins

TIRAP (also known as MAL) gene is located at chromosome 11q24.2. Two isoforms of TIRAP are identified so far; one contains 221 amino acids, whereas the other isoform has 235 residues (Fig. 1), which is commonly found in human. TIRAP has a PBD domain, which binds PtdIns(4,5)P$_2$ at the plasma membrane and PtdIns(3)P at the
endosomal membranes, and a C-terminal TIR domain that is responsible for association with TLRs and MyD88.

Kagan and Medzhitov reported that a minimal region of 21 residues (15-35) within the N-terminal PBD of TIRAP is necessary for TIRAP’s membrane binding [46], which is termed the PIP-binding motif (PBM). They also identified four key PIP-binding residues in PBM, including Lys15, Lys 16, Lys 31, and Lys 32, mutations of which inhibit TIRAP’s binding to PtdIns(4,5)P$_2$ at the plasma membrane. Another residue within PBM, Thr28, is reported as a phosphorylation site by IRAK1 and IRAK4. Phosphorylation of TIRAP by IRAKs, followed by ubiquitination and degradation, represent a negatively regulation of TLR4/TLR2 signaling[19]. The N-terminal PBD contains a proline, glutamic acid, serine and threonine motif so-called the PEST motif (residues 40-43), which often found in short-lived proteins that undergo phosphorylation, polyubiquitination of lysine residues and targeted degradation via the 26S proteasome [47]. It was reported that suppressor of cytokine signaling-1 (SOCS-1) binds to PEST in TIRAP PBD through its SH2 domain and that mediates polyubiquitination of TIRAP on two lysine residues (Lys 15 and Lys 16) within PBM.

The TIRAP TIR domain (residues 79-221) contains five-stranded parallel $\beta$-sheet ($\beta$A-$\beta$E) surrounded by four ($\alpha$A, $\alpha$C- $\alpha$E) $\alpha$-helices that were found in other TLR TIR domains. It lacks a helical element $\alpha$B between $\beta$B and $\beta$C, resulting a long loop AB connecting the first helix ($\alpha$A) and the second $\beta$-strand ($\beta$B), instead of a BB loop. The
folding conformation is further stabilized by two disulfide bonds (residues C89-C134 and C142-C174) [48]. The unique extra long AB loop is integral for binding to MyD88 TIR and TL4-TIR. In a nutshell, the AB loop is the bridge that connects MyD88 and TLR4 [49]. Tyrosine residues 86, 106, and 187 in the TIR domain of TIRAP are the phosphorylation sites of the tyrosine kinase Bruton’s tyrosine kinase. Mutation of these residues inhibits TLR2- and TLR4 signaling [50]. TIRAP TIR domain has a TRAF6 binding site at amino acid positions 188-193 that allows the recruitment of TRAF6 to the plasma membrane [51].

2.3 The role of TIRAP in TLR and other signaling pathways

2.3.1 TIRAP in the MyD88-depedent signaling

TIRAP’s primary function was described as a bridge to recruit cytosolic MyD88 to TLR TIR dimers. In resting cells, TIRAP was found residing in the plasma membrane of professional and non-professional cells. When specific TLRs (i.e., TLR2 and TLR4) become active by bacterial components (i.e., LPS), they dimerize, and move laterally to reach PtdIns(4,5)P$_2$-rich domains at the plasma membrane [52], where TIRAP locates at, and associate with it through TIR-TIR interactions, which is likely the first event in the cytosol [53]. The receptor-TIRAP complex may then recruit MyD88 and IRAKs (IRAK1/2, IRAK4) through TIR domain and DD-DD interactions, thus, leading to the formation of the protein complex so-called myddosome, which consists of 6 MyD88, 4 IRAK1/2, and 4 IRAK4 [21]. This complex formation is required to propagate signaling to downstream effectors to trigger pro-inflammatory responses. The ability of TIRAP to recruit and stabilize the formation of myddosome is dependent on its modular structure, once the
membrane is anchored via PtdIns(4,5)P$_2$, spatial localization facilitates and stabilizes the TLR-TIRAP-MyD88 complex [54]. Mutations in the PtdIns(4,5)P$_2$ binding residues in TIRAP or the hydrolysis of cellular PtdIns(4,5)P$_2$ by the PtdIns(4,5)P$_2$ phosphatase abrogates the recruitment of TIRAP to the plasma membrane as well as its ability to induce cytokine synthesis in response to LPS [46, 55].

TIRAP was previously excluded as an adaptor for endosomal TLRs, such as TLR9 [56]. However, Bonham et al showed that TIRAP promotes the assembly of the myddosome at both the cell surface and endosomal membranes; confocal microscopy data showed that TIRAP can localize to both PtdIns(4,5)P$_2$-rich subdomains at the plasma membrane and PtdIns(3)P-rich subdomains in endosomal membranes [16]. Macrophage with PtdIns(4,5)P$_2$-localized TIRAP responses to LPS, a TLR4 ligand, whereas macrophage with PtdIns(3)P-localized TIRAP responses to CpG DNA, which is a ligand of endosomal TLR9 [16]. These data indicate that TIRAP has a widespread role in TLR signaling transduction.

2.3.2 TIRAP in MyD88 bridging-independent signaling

Emerging evidence show that TIRAP is more than just a bridge to MyD88. Recent works show that TIRAP is involved in MyD88-independent Rac1-PI3K-Akt NF-$\kappa$B transactivating pathway and interferon-gamma (IFN-$\gamma$) receptor (IFNGR) signaling [57] as shown in detail below.

2.3.2.1 TIRAP in parallel pathways of NF-$\kappa$B nuclear translocation and transactivation
Transactivation of NF-κB subunit p65 involves the serine phosphorylation of its transactivation 1 domain by PI3K-Akt kinase, a study demonstrated TLR2-Rac-1 function as a activator of PI3K-Akt [58]. Rac1 activates NF-κB through PI3K-mediated p65 transactivation to allow its nuclear translocation [59]. Another study reported LPS induced Rac1-dependent NF-κB activation, indicating that TLR4 plays a role in propagating the Rac1-PI3K-Akt pathway [60]. Similar results were obtained by LPS stimulation of human microvessel endothelial cells that induce Rac1, and that negative expression of TIRAP, but not MyD88, blocked Rac1-induced regulation of the HIV-long terminal repeat, indicating a role of TIRAP in Rac-PI3K-Akt NF-κB transactivating pathway and in HIV replication modulation [60].

TIRAP has a putative TRAF6 binding site in a sequence spanning residues, 188-193 [61], and it co-immunoprecipitates with TRAF6, whereas a mutant of TIRAP, TIRAP E190A, inhibited TLR2 or TLR4 induction of NF-κB signal. Verstak and colleagues confirmed this observation by demonstrating TIRAP binds TRAF6 and recruits it to the plasma membrane [51]. They also described that association between TIRAP and TRAF6 is required for the serine phosphorylation of p65, thus, indicating a mechanism of TIRAP-dependent NF-κB transactivation.

2.3.2.2 TIRAP plays a role in IFNGR signaling

Cheallaigh et al show that TIRAP has a TLR-independent role in IFNGR signaling. In response to IFN-γ, p38 MAPK will be phosphorylated in macrophages; however, this was impaired in TIRAP−/− macrophages, and IFN-γ induced IFN-inducible protein 10 (IP10) secretion and autophagy was reduced in TIRAP−/− macrophages. These data
demonstrate that TIRAP was required for IFN-γ induced P38 MAPK phosphorylation, autophagy and IP-10 secretion. They also reported that TIRAP directly interacts with IFNGR and recruits MyD88 to IFNGR by co-immunoprecipitation and fluorescence lifetime imaging microscopy-fluorescence resonance energy transfer. They observed increased recruitment of MyD88 to IFNGR in IFN-γ treated cells; however, this interaction was reduced to background level in TIRAP-deficient cells [57]. These data demonstrate that TIRAP is required for the interaction between MyD88 and IFNGR, indicating a MyD88-bridge dependent role of TIRAP outside of TLR signaling.

2.4 TIRAP associates with human diseases

A single nucleotide polymorphism (SNP) of TIRAP, TIRAP S180L has been reported to be protective against several diseases by a number of studies. The gene of TIRAP may have this polymorphism as homozygous ser/ser or leu/leu or heterozygous as ser/leu. Khor et al. analyzed 33 SNPs in TIRAP in several populations, and found that TIRAP S180L is present in UK, Vietnamese, and African populations. They described that, in comparison with homozygote S180S and L180L, heterozygote S180L associate with protection from pneumococcal disease, bacteremia, malaria, and tuberculosis (TB) [62]. Their conclusion was confirmed later by a number of studies [57, 63, 64]. Cheallaigh et al. demonstrate that TIRAP S200L, a murine equivalent of human TIRAP S180L, is associated with TB in vivo. In contrast to homozygote, heterozygote S180L mice were protected from weight loss, a feature of TB, indicating that TIRAP S180L conferred protection from TB disease.
There are some controversies about the association of TIRAP S180L with TB. A study conducted by Nejentsev et al. show evidence proving that no association between TIRAP S180L with TB in Russian, Ghanaian, and Indonesian populations [65]; later on, a meta-analysis showed the same result [66]. However, their studies are on different population and they use different statistical methods from Khor’s work. Furthermore, Nejentsev et al did not conduct study on other three diseases [65]. Although the controversy on TB, this SNP is associated with malaria, sepsis, leprosy, rheumatoid arthritis, and chronic Chagas cardiomyopathy [57, 62, 64, 67-69].
PART III. THE ROLES OF PHOSPHOINOSITIDES IN TLR SIGNALING

3.1 Introduction to phosphoinositides

Phosphoinositides (PIPs), are phosphorylated derivatives of phosphatidylinositol, which exist in the cytosolic face of plasma membrane and cell compartments and nucleoplasm. They compose a small fraction of cellular phospholipids, yet play critical roles in nuclear function, membrane trafficking, cytoskeletal dynamics, and signal transduction [70-72]. There are seven known PIPs, through interaction with kinase or phosphatase mediated by their head groups, which can be reversibly phosphorylated (Fig. 2). Numerous studies have described that each PIP has a unique cellular distribution. For example, PtdIns(4,5)P$_2$, PtdIns(3,4)P$_2$, and PtdIns(3,4,5)P$_3$ are mainly localized at the plasma membrane; while PtdIns(3)P and PtdIns(3,5)P$_2$ are often found in endosome membranes; and PtdIns(4)P concentrates in the Golgi Apparatus (Table 3) [73].

PIPs interact with signaling proteins that either reside in the membrane, or get recruited to the membrane. They bind to specific domain of a signaling molecule. For example, the
epsin N-terminal homology (ENTH) and phosphotyrosine-binding (PTB) domains specifically bind to PtdIns(4,5)P₂, the FYVE and PX domains bind to PtdIns(3)P, and so on (Table 3). Typically, binding of proteins to PIPs involve an electrostatic interaction with the negative charges of the phosphate group localized in the inositol ring. These associations are the foundation of signal transduction from the membrane to cytosol.

Table 3: Phosphoinositide-binding domains and their subcellular localization.

<table>
<thead>
<tr>
<th>PIPs</th>
<th>Relative Abundance</th>
<th>Localization</th>
<th>Binding Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtdIns(3)P</td>
<td>Low</td>
<td>Endosome, Lysosome</td>
<td>FYVE, PX</td>
</tr>
<tr>
<td>PtdIns(4)P</td>
<td>High</td>
<td>Golgi Apparatus</td>
<td>PH</td>
</tr>
<tr>
<td>PtdIns(5)P</td>
<td>Low</td>
<td>Nucleus</td>
<td>PHD</td>
</tr>
<tr>
<td>PtdIns(3,4)P₂</td>
<td>Low</td>
<td>Plasma Membrane</td>
<td>PH, PX</td>
</tr>
<tr>
<td>PtdIns(3,5)P₂</td>
<td>Low</td>
<td>Endosome</td>
<td>GRAM, ENTH</td>
</tr>
<tr>
<td>PtdIns(4,5)P₂</td>
<td>High</td>
<td>Plasma Membrane, Nucleus</td>
<td>PH, ENTH, PTB</td>
</tr>
<tr>
<td>PtdIns(3,4,5)P₃</td>
<td>Low</td>
<td>Plasma Membrane</td>
<td>PH, GLUE</td>
</tr>
</tbody>
</table>

FYVE: Named after the four proteins in which they were first identified, Fab1, YOTB/ZK632, Vac1p, and EEA1; PX: Phox homology; PH: Pleckstrin homology; PHD: Plant homeodomain; GRAM: Glucosyltransferase, Rab-like GTPase activators and Myotubularins domain; ENTH: epsin NH₂-terminal homology; PTB: Phosphotyrosine-binding domain; GLUE: GRAM-Like Ubiquitin-binding in EAP45

3.2 PtdIns(4,5)P₂ and its role in the TLR signaling pathway

PtdIns(4,5)P₂ is the best studied and one of two most abundant PIPs, with the other being PtdIns(4)P, together, they weigh 0.2-1% of total cellular phospholipids [74]. Based on a study, PtdIns(4,5)P₂ density in plasma membrane was reported to range between 5,000-20,000 molecules/µm² [75]. PtdIns(4,5)P₂ is produced by the phosphorylation of either PtdIns(4)P or PtdIns(5)P (Fig. 2). Since there is only very little PtdIns(5)P in cells, most PtdIns(4,5)P₂ is generated from PtdIns(4)P. PtdIns(4,5)P₂ can be hydrolyzed by either phospholipases or phosphatases. Cleavage by phospholipases, such as phospholipase C (PLC) and phospholipase A2 (PLA2), produce metabolites that propagate and amplify
signaling, whereas dephosphorylation, primarily by 5-phosphatases, controls steady-state levels of PtdIns(4,5)P$_2$ and turns off its signaling. Besides hydrolysis, PtdIns(4,5)P$_2$ can be converted to PtdIns(3,4,5)P$_3$ by a kinase, or to PtdIns(4)P and PtdIns(5)P by phosphatases. PtdIns(4,5)P$_2$ is mainly localized to the plasma membrane and nucleus [76-78]. PtdIns(4,5)P$_2$ participates in nearly all events that occur at the cell surface, including exocytosis, endocytosis, phagocytosis, ion channel-mediated transportation, signal transduction, cell adhesion, and cell motility [79].

PtdIns(4,5)P$_2$ is required for the MyD88-dependent TLR4 pathway. It is present at a low levels at plasma membrane at a rest state condition; however, with extracellular stimuli, it increases rapidly [79]. With activation of TLR4 by LPS, the ADP-ribosylation factor 6 and β2 integrin may stimulate PIP5K for PtdIns(4,5)P$_2$ production at certain membrane subdomains [46], thus, concentrating TIRAP through its interaction with its PBM for TLR4-TIRAP-MyD88 complex formation, and leading to TLR4 signaling from the plasma membrane. Then PtdIns(4,5)P$_2$ is degraded by PLCγ [80], and this may attenuate MyD88-dependent signaling. Thus, degradation of PtdIns(4,5)P$_2$ may function as a molecular switch for TLR pathway. Yet, the detailed molecular mechanism by which PtdIns(4,5)P$_2$ metabolism modulates TLR signaling is unknown.
CHAPTER II: RESEARCH GOALS

In this thesis, I will show findings that are associated with the function and modulation of membrane binding of TIRAP in the TLR signaling pathway at the structural level. Accumulated evidence indicates that TLR4 must be delivered to heterogeneous membrane regions, perhaps PtdIns(4,5)P$_2$-rich regions [52], to initiate signal transduction [81, 82]. These results led us to focus on the investigation of the structural basis of PtdIns(4,5)P$_2$ recognition by TIRAP since this association is likely to be the earliest cytosolic events, and how this association is regulated. These results will provide the basis to understand why PtdIns(4,5)P$_2$ is preferred over other negatively charged lipids, how critical TIRAP’s membrane insertion in this process is, and under what condition TIRAP is compartmentalized at the plasma membrane to trigger signaling. The specific aims were:

Aim 1. To characterize PtdIns(4,5)P$_2$ recognition and binding site on TIRAP PBM

This specific aim was designed to biophysically characterize the binding of TIRAP PBM to its binding partners, PIPs, especially PtdIns(4,5)P$_2$. Firstly, I biophysically demonstrated that TIRAP PBM binds PtdIns(4,5)P$_2$. Secondly, the secondary structure of TIRAP PBM was investigated using circular dichroism (CD) analysis. To detect the structural properties of TIRAP PBM under physiological condition, I used dodecylphosphocholine (DPC) micelles as membrane mimics. Different conformations of TIRAP PBM in the absence and presence of DPC micelles were found and further confirmed by Nuclear Magnetic Resonance (NMR) studies. Thirdly, PtdIns(4,5)P$_2$ interacting residues on TIRAP PBM were mapped by two-dimensional (2D) NMR
spectroscopy, and mutagenesis results confirmed the importance of key PtdIns(4,5)P$_2$ interacting residues. Binding of TIRAP PBM to PtdIns(3)P, inositol trisphosphates, and phosphatidylinositol (PI) were also tested to study the role of head group and acyl chains of PtdIns(4,5)P$_2$.

Aim 2. To further define the structural basis and PtdIns(4,5)P$_2$ binding properties of TIRAP PBM.

To better understanding the binding mechanism of TIRAP PBM to membrane PIPs, the tertiary structure of TIRAP PBM in DPC micelles was solved using 2D and 3D NMR spectroscopy. 2D $^1$H NOESY and 2D $^1$H TOCSY spectra were collected using a 600 MHz NMR instrument at Virginia Tech. Nuclear Overhauser effect (NOEs) and chemical shifts were obtained to estimate the 3D structure of the peptide. Nuclear spin relaxation (NSR) experiments were used to further substantiate the structural results and investigate conformational changes of the peptide in the presence of PtdIns(4,5)P$_2$.

Then, the location of TIRAP PBM relative to the micelle surface was determined by paramagnetic studies. Lastly, PtdIns(4,5)P$_2$ binding affinity with TIRAP PBM was detected using far-UV CD spectroscopy.

Aim 3. To investigate the modulation of TIRAP binding to PIPs

Previous work showed a phosphorylation site at TIRAP’s Thr28 residue, located within PBM [19]. Phosphorylation, followed by ubiquitination and proteasome degradation, represents a negative regulation of TIRAP. Therefore, we mimicked phosphorylation by mutating Thr28 to Asp (T28D) in TIRAP and TIRAP PBM to study its function. It demonstrated that mutation of Thr28 to Asp inhibited both TIRAP and its PBM’s binding
to PIPs. To address the structural characteristics of the T28D mutated, eventually the phosphorylated TIRAP, secondary structures of TIRAP PBM T28D and the synthetic Thr28-phosphorylated TIRAP PBM (TIRAP PBM-P) were compared with the wild-type peptide. The results revealed that the mutation distorted the helical conformation in TIRAP PBM. To further look into the function relevance of Thr28 in TIRAP in a physiological condition, I investigated the subcellular localization of the defective version of TIRAP generated in this study, including TIRAP T28D and TIRAP K16A/K31A/K32A in HEK293 cells.
CHAPTER III: MATERIALS AND METHODS

1.1 Materials

The following is a list of chemicals used and their suppliers:

Restriction endonucleases: EcoRI and BamHI, (NEB), DNA ligation kit (Takara, Japan), acetonitrile (Fisher Scientific), DL-dithiothreitol (DTT), isopropyl-β-D-thiogalactopyranoside (IPTG), benzamidine HCl (Research Products International), and Luria Bertani (LB) medium (Research Products International), lysozyme (MP Biomedicals), Triton X-100 (EMD Chemicals), glutathione Sepharose 4B beads (GE Healthcare), bovine thrombin (MP Biomedicals), \(^{15}\)N ammonium chloride, \(^{13}\)C glucose, deuterium oxide, and \(d_{58}\)-dodecylphosphocholine (Cambridge Isotopes Laboratories), N-dodecylphosphocholine (Anatrace/Affymetrix), PtdIns(4,5)P\(_2\) di C8, PtdIns(4,5)P\(_2\) di C16, PtdIns(3)P di C8, phosphatidylinositol di C8, inositol 1,4,5-trisphosphate (IP\(_3\)) (Echelon Lipids), Rabbit anti-GST antibody (Santa Cruz Biotech), 16-doxyl steric acid (16-DSA), 5-DSA (Sigma-Aldrich), manganese chloride (J. T. Baker), HEK293 cells (ATCC), Dulbecco’s minimal essential medium (DMEM, Mediatech, Corning subsidiary), fetal bovine serum (FBS, Mediatech, Corning subsidiary), lipofectamine reagent (Invitrogen), rhodamine phalloidin (Life Technologies), 4’, 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich), Prolong Gold Antifade Mountant (Thermo Fisher Scientific), A phosphorylated TIRAP PBM was synthesized by the Protein Synthesis Facility at Tufts University. All other chemicals were of analytical reagent grade.

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1.2 Cloning, Expression, and Purification

Human TIRAP and TIRAP PBM cDNA (representing residues 15-35) were cloned into a pGEX4T1 plasmid. TIRAP was also cloned into a pCS2-EGFP-LIC vector. Site-directed mutagenesis was introduced in TIRAP and PBM cDNA constructs using the Quick Change method. The recombinant plasmids were transformed into *Escherichia coli* (Rosetta) for their expression. Then, bacteria cells were grown in LB media at 37°C until cells reached an optical density of 0.8. The overexpression of GST fusion protein was induced by addition of 0.5mM IPTG for 4 h (TIRAP) and 2 h (TIRAP PBM) at 25°C. $^{15}$N- and $^{13}$C-labeled proteins were expressed in minimal media with $^{15}$N-labeled ammonium chloride and $^{13}$C-U-glucose as nitrogen and carbon sources, respectively. GST fusion proteins were purified using glutathione beads. The GST tag was removed by incubation with bovine thrombin for 3 h at room temperature, and the digestion was stopped by addition of 1mM DTT. TIRAP PBM (2686 Da) was further purified using a methodology we reported previously [83].

1.3 Protein-Lipid Overlay Assay

Serial dilutions of PtdIns(4,5)P$_2$/PtdIns(3)P di-C16 were spotted onto a nitrocellulose membrane to which they attach, and dried for 1 h at room temperature followed by blocking with 3% fatty acid-free BSA (Sigma) for 1 h at room temperature. Afterwards, membranes were incubated with 1 μg/mL GST fusion proteins at 4°C overnight in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 8.0. Followed by four washes with the same buffer, bound proteins were probed with rabbit anti-GST antibody (Santa
Cruz Biotech) and donkey anti rabbit-horse radish peroxidase (GE Healthcare). Protein binding was probed using West Pico chemiluminescent reagent (Pierce).

1.4 Circular Dichroism Spectroscopy
Far UV (190-260 nm) CD spectra were recorded using a Jasco J-815 spectropolarimeter. The spectra of 20 μM TIRAP PBM, its mutants (TIRAP PBM K16A/K31A/K32A, T28D, T28A), and TIRAP PBM-P, in 5 mM Tris-HCl (pH 7.0), 40 mM KF, were recorded in the absence and presence of DPC and c8 PtdIns(4,5)P₂ or other PIPs. Five CD spectral accumulations were collected at 1-nm bandwidth with a response time of 1s and at a scan speed of 50 nm/min. Buffer spectra were also obtained under the same experimental conditions and subtracted from the protein spectra before analysis.

1.5 NMR Spectroscopy
NMR experiments were performed at 25°C on a Bruker 600-MHz spectrometer equipped with a TBI probe (Virginia Tech). 2D \(^1\)H-\(^{15}\)N HSQC (Heteronuclear Single Quantum Coherence) spectroscopy was used for titration of TIRAP PBM by its ligand c8 PtdIns(4,5)P₂. The spectra of 75 μM \(^{15}\)N labeled TIRAP PBM in 90% H₂O/10% \(^2\)H₂O, 10 mM \(d_4\)-citric acid (pH 6.0), 50 mM KCl, 1 mM NaN₃ (NMR buffer), and 50 mM \(d_{18}\)DPC were collected in the presence of increasing ratio of c8 PtdIns(4,5)P₂ or other phospholipids, and processed with Topspin and NMRPipe [84], and analyzed by Sparky [85]. The chemical shift perturbations were calculated using the following equation [86]:

\[ \Delta \sigma = \frac{\sigma_{obs} - \sigma_{free}}{\sigma_{free}} \]

where \(\Delta \sigma\) is the chemical shift perturbation, \(\sigma_{obs}\) is the observed chemical shift, and \(\sigma_{free}\) is the free chemical shift
\[ \Delta \delta(^{1}H, ^{15}N) = [(\Delta \delta \, ^{1}H)^2 + (\Delta \delta \, ^{15}N)^2]/6^{0.5} \]

Resonance assignment were obtained by triple resonance experiments acquired using the same NMR spectrometer at Virginia Tech. One mM \(^{15}N\)-, \(^{13}C\)- labeled TIRAP PBM in NMR buffer with 130mM \(d_{38}\)DPC was used. \(^1H\) chemical shifts were referenced using sodium 4,4-dimethyl-4-silapentane-1-sulfonate. Sequential assignments of the backbone of TIRAP PBM were made from \(^1H\), \(^{15}N\)-HSQC, CBCA(CO)NNH, HNCACB, and HNCO experiments. All spectra were processed using NMR Pipe [84] and \(^1H\), \(^{15}N\), and \(^{13}C\) resonance assignments were determined using the CCPNMR software [87]. Resonance of assignments will be deposit at the Biological Magnetic Resonance data Bank (http://www.bmrb.wisc.edu).

Protein for structure determination was prepared as 0.3-1 mM in NMR buffer with 130 mM \(d_{38}\)DPC. 2D \(^1H\) TOCSY and 2D \(^1H\) NOESY were acquired at 25°C using a 900 MHz Bruker Avance III NMR instrument at the University of Colorado, Boulder by Dr. Geoffrey Armstrong. Spectra were then processed and interpreted to obtain NOEs, hydrogen bonds, and residual dipolar coupling restraints. One hundred forty five NOE-derived internuclear constraints were used to calculate the structure using Rosetta, and the quality of the structures was assessed using Procheck software [88, 89]. The final structures were generated using PyMOL. The electrostatic potential of TIRAP PBM was estimated using the Adaptive Poisson-Boltzmann Solver program, which was plugged in PyMOL. NSR experiments were conducted using an 800 MHz Bruker NMR instrument at the University of Virginia by Dr. Jeffrey Ellena. Protein samples were prepared as 0.15 mM with 100 mM \(d_{38}\)DPC in NMR buffer with or without 1.8 mM PtdIns(4,5)P\(_2 \) di C8.
(12-fold). Both NOE and products of \( R_1 \) (longitudinal relaxation rates) and \( R_2 \) (transverse relaxation rates) were calculated and analyzed [90].

Paramagnetic studies were used to determine the location of TIRAP PBM relative to the micelle surface. Three different paramagnetic agents 16-DSA, 5-DSA, and MnCl\(_2\), were employed. Both 16-DSA and 5-DSA were dissolved in \( d_4\)-methanol due to its insolvability in aqueous solvent. 0.1-2 mM paramagnetic agents were added to the \(^{15}\text{N-}\)TIRAP PBM (0.2 mM) in NMR buffer containing 50 mM \( d_{38}\)DPC. The intensity reduction of \(^1\text{H}-^{15}\text{N}\) HSQC spectra caused by line broadening because of close proximity to the spin label was calculated as the ratios of the peak intensities of the spectra in the presence to the absence of the probe.

1.6 Cell Culture and Staining

HEK293 cells were grown in DMEM supplemented with 10% (v/v) FBS in 37°C humidified incubator in the presence of 5% CO\(_2\). Cells were split every 2-3 days to maintained at about 50% confluence. Transient transfection of TIRAP (in pCS2-EGFP) and its mutant plasmids were performed using the Lipofectamine reagent at a ratio of 2.5 to DNA (0.4 μg DNA, 1μM Lipofectamine) in OptiMEM reduced serum medium for 2x10\(^5\) cells. Cells grown on grid-glass coverslips were fixed in 4% formaldehyde for 10 min, and permeabilized by 0.1% Triton X-100 in PBS for 5 min at room temperature, and blocked with 1% BSA (Sigma) in PBS at 4°C overnight. Five units/ml rhodamine phalloidin and 0.1μg/ml DAPI were used to stain the cytoskeleton (actin stress fiber)
and nuclei, respectively. Coverslips then were mounted onto glass slides with Prolong Gold Antifade Mountant.

1.7 Confocal Microscopy

The cell images were taken using confocal microscope (Biocomplexity Institute, Virginia Tech). GFP fusion TIRAP and its mutants were excited at 488 nm and their emissions were captured using a 505-550 nm broad pass filter. Rhodamine phalloidin and DAPI was excited at 543 nm and 405 nm, respectively. The detector gain measurements were under 750. All images are representative of at least 8 scans.
CHAPTER IV: RESULTS

1.1 PBM IS SUFFICIENT AND NECESSARY FOR TIRAP’S PHOSPHOINOSITIDE BINDING

Previous work showed that TIRAP has a preference to binds PIPs in membranes, and PBM is required for TIRAP’s membrane targeting [46]. Association of TIRAP PBM to PtdIns(4,5)P$_2$ was detected by the protein-lipid overlay assay (PLOA). The results show both TIRAP and TIRAP PBM bind to PtdIns(4,5)P$_2$, but not the negative control, GST (Fig. 3), indicating that PBM plays a critical role in TIRAP’s PIP binding.

![Figure 3. TIRAP PBM interacts with PtdIns(4,5)P$_2$. Lipid-protein overlay assay of the indicated proteins with immobilized PtdIns(4,5)P$_2$. TIRAP and TIRAP PBM (10 μg) were incubated with immobilized PIP2. GST and Dab2 N-PTB domain represent the negative and positive controls, respectively.](image)

1.2 TIRAP PBM ADOPTS A HELICAL CONFORMATION IN THE PRESENCE OF MEMBRANE MIMICS, SUCH AS DPC MICELLES

The pivotal function of PBM in TIRAP’s membrane targeting draw our attention to its structure. Therefore, untagged TIRAP PBM was purified and its secondary structure was tested by CD spectroscopy. The results show that TIRAP PBM displayed a minimum at 200 nm in the far-UV CD region, characteristic of unstructured protein spectra. The fact that TIRAP PBM is necessary and sufficient for membrane binding [46]
led us to hypothesize that it may change its conformation by the addition of membrane mimics. A list of membrane mimics was screened, and results show that TIRAP PBM displayed conformational changes in both DPC (Fig. 4A) and lyso-myristoylphosphatidylglycerol (LMPG) micelles (data not shown). However, micelles have limitations as membrane mimics. They are curved single layers, unlike biological membranes, which are bilayers. However, from a solution NMR perspective, large membrane mimetic, such as liposomes, associate with protein as a part of larger complex, leads to signal broadening, reducing spectral resolution. Thus, detergent micelles, as the smallest membrane mimics, represent an approximate system for membrane protein studies using NMR. Therefore, we used DPC micelle for most of our NMR studies.

A $^1$H-$^{15}$N HSQC spectrum of TIRAP PBM, in the presence of DPC micelles, exhibits

![Figure 4. TIRAP PBM folds in the presence of DPC micelles.](image)

Left. TIRAP PBM (20 μM) CD spectra was collected in the absence (solid line) and presence of DPC micelles (dotted line). Right. TIRAP PBM (75 μM) HSQC spectra was also collected in the absence (black) and presence of deuterated DPC micelles (red).
more dispersed chemical shifts than that in the absence of DPC micelles (Fig. 4B), indicating a different conformational state, consistent with the far-UV CD results. The secondary structure of TIRAP PBD (residues 1-85) was also studied in the absence and presence of membrane mimics, by CD and NMR; however, the results showed, in all cases, a disordered structure (data not shown).

1.3 PHOSPHOINOSITIDE INDUCE SECONDARY STRUCTURE IN TIRAP PBM

It was reported that PtdIns(4,5)P$_2$ can induce helical conformation in PtdIns(4,5)P$_2$ binding proteins [91]. As TIRAP PBM is a PtdIns(4,5)P$_2$ binding motif, we hypothesized that PIPs, such as PtdIns(4,5)P$_2$ can induce TIRAP PBM’s structural changes. Far-UV CD spectra of TIRAP PBM with increasing amount of PtdIns(4,5)P$_2$, PtdIns(3)P, phosphatidylinositol, and IP$_3$ (the head group of PtdIns(4,5)P$_2$) were collected. PtdIns(4,5)P$_2$ binding to TIRAP PBM induced conformational changes with only 1-fold of ligand (20 μM), and saturated at 8-fold of PtdIns(4,5)P$_2$ with an apparent $K_D$ of ~25 μM (Fig. 5A and E); PtdIns(3)P also induced TIRAP PBM’s folding at low micromolar concentrations, and saturated at 16-fold (Fig. 5B); However, neither monodispersed phosphatidylinositol (PI) nor IP$_3$ were able to trigger TIRAP PBM’s folding (Fig.5C and D). Micellar PI was able to induce PBM’s folding, which is consistent with the CD data of TIRAP PBM using DPC micelles. These results suggest that the interaction of TIRAP PBM with PIPs requires hydrophilic interactions with the head group, as well as with the acyl chains of the lipid.
Figure 5. PIPs induce helical structure of TIRAP PBM. A. PtdIns(4,5)P$_2$ is able to induce helical structure of TIRAP PBM. Far UV CD spectra of TIRAP PBM (20 μM) were superimposed and color-coded as shown in the set according to the concentration of dioctanoyl PtdIns(4,5)P$_2$. B. The head group only of PtdIns(4,5)P$_2$ is insufficient for TIRAP PBM's helical structure formation. C. Low concentration of PI is not enough to induce helical structure formation, but micellar concentration of PI is able to induce helical structure. D. PtdIns(3)P is able to induce helical structure of TIRAP PBM. E. Plot of the ellipticity at 222 nm of TIRAP PBM with addition of PtdIns(4,5)P$_2$. 
1.4 IDENTIFICATION OF TIRAP’S KEY PHOSPHOINOSITIDE-BINDING RESIDUES

NMR spectroscopy was used to map the PIP-binding site in PBM. In a \(^{1}\text{H}^{15}\text{N}\) HSQC spectrum, each residue (except proline) give rise to one signal (chemical shift) that corresponds to an amide group of the protein. These chemical shifts can be tracked by perturbations in their chemical environments to interaction with physiological ligands or even changes in structure or dynamics. Highly resolved \(^{1}\text{H}^{15}\text{N}\) cross peaks are evident in the spectra indicating the presence of a soluble peptide. Chemical shift perturbations of the residues Lys16, Lys31, and Lys32 were observed after stepwise addition of PtdIns(4,5)P\(_2\) di C8 (Fig. 6A, C), suggesting that their side chains are strong candidates for directly contacting the phosphate groups of the lipid. Perturbations of the nonpolar residues Leu18 and Leu30 were also detected (Fig. 6C), indicating that they may form hydrophobic interactions with the lipid. Interaction of TIRAP PBM with PtdIns(3)P, PI, and IP\(_3\) were also evaluated. PtdIns(3)P titration of TIRAP PBM shows similar interacting residues, such as Leu18, Lys31, and Lys32, but perturbation were less evident compared with PtdIns(4,5)P\(_2\) titrations; PI promotes very weak chemical shift perturbations of TIRAP PBM (Fig. 6E and G); no chemical shift perturbations were observed when IP\(_3\) was tested under same experimental conditions (Fig. 6F, H).
Figure 6. TIRAP PBM interacts with PIPs in the presence of DPC micelles. $^1$H, $^{15}$N HSQC spectra of uniformly $^{15}$N-labeled DPC-embedded TIRAP PBM (75 μM) were superimposed and color-coded as shown in the inset according to the concentration of di-c8 PtdIns(4,5)P₂ (A), PtdIns(3)P (B), PI (E), IP₃ (F) in DPC micelles. The resonances of amino acids displaying chemical shift changes upon PIPs binding are boxed. C, D, G, H, Perturbation of chemical shifts of TIRAP PBM in HSQC spectra.
To further confirm PtdIns(4,5)P² interacting residues in TIRAP PBM, K16A, K31A/K32A, and K16A/K31A/K32A mutants were generated and their PIP-binding properties tested using the PLOA. As shown in Fig. 7, K16A and K31A/K32A mutants of TIRAP PBM exhibited reduced association to the PIP, but a combination of all these mutations dramatically inhibited binding to PtdIns(4,5)P².
and PtdIns(3)P. These mutations exhibited similar effects when full-length TIRAP was tested (Fig. 8), supporting the data that the effect of the mutations designed on PBM can be visualized in the full-length protein.

1.5 SOLUTION STRUCTURE OF TIRAP PBM SHOWS A CENTRAL ALPHA HELIX AND TWO FLEXIBLE TERMINI

To precisely pinpoint the lipid-binding site in TIRAP PBM, the solution structure of TIRAP PBM in DPC micelles was solved using NMR spectroscopy (Fig. 9A-B and Table 4). 2D $^1$H NMR spectroscopy is a useful method for investigating a structure of the peptide in membrane environments for biophysical studies [92, 93]. Distance restraints were derived from homonuclear 2D NMR experiments. One hundred forty-five NOE-derived internuclear constraints were used in the calculations (Table 4). A total of twenty lowest energy structures were assessed using Procheck software [88, 89]. As shown in Fig. 9C, the solution NMR structure of TIRAP PBM in DPC micelles shows a central short helical element, spanning residues Leu18-Leu30, a region that is well conserved in primates (Fig. 7C). The solution structure also shows that both N- and C- termini are flexible, and that all the key PtdIns(4,5)P$_2$-interacting residues (Lys16, Leu18, Leu29, Lys31, and Lys32) identified by NMR analysis face one side, suggesting that these residues may form a binding site for PtdIns(4,5)P$_2$.

The solution structure reveals a possible salt bridge formed by the side chain of Asp23 and Arg26 that facing along with the two flexible termini (Fig. 12A). The estimated electrostatic potential of DPC-embedded TIRAP PBM shows positively charged surface
(Fig. 9D), confirming the charge-dependent nature of the interaction. NSR data of TIRAP PBM/DPC were also collected in the absence and presence of PtdIns(4,5)P$_2$. Both heteronuclear NOE and products of R$_1$ and R$_2$ were calculated. The NSR results are consistent with solution structure, shows a central ordered structure, and two disordered termini (Fig. 9E-F). Interestingly, the N- and C-termini displayed a more ordered structure in the presence of PtdIns(4,5)P$_2$ (Fig.9E, F).

Table 4. NMR and refinement statistics for TIRAP PBM. NMR structural statistics for the lowest energy conformers of TIRAP PBM using the Protein Structure Validation Suite.

<table>
<thead>
<tr>
<th>NMR Distance and Dihedral Constraints</th>
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<tbody>
<tr>
<td>Dihedral angle restraints</td>
<td></td>
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<tr>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>$\phi$</td>
<td>28</td>
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<tr>
<td>$\psi$</td>
<td>28</td>
</tr>
<tr>
<td>Structure statistics</td>
<td></td>
</tr>
<tr>
<td>Dihedral angle constraints (º)</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Max. dihedral angle violation</td>
<td>7.3 ± 3.8</td>
</tr>
<tr>
<td>Deviations from idealized geometry</td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.007</td>
</tr>
<tr>
<td>Bond angles (º)</td>
<td>0.4</td>
</tr>
<tr>
<td>Average pairwise r.m.s. deviation (Å)$^a$</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
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<tr>
<td>Heavy</td>
<td>0.6</td>
</tr>
<tr>
<td>Backbone</td>
<td>0.3</td>
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$^a$Pairwise backbone and heavy-atom r.m.s. deviations were obtained by superimposing residues 15-35 of TIRAP PBM among 20 lowest energy refined structures.
Figure 9. Solution structure and dynamics of TIRAP PBM in DPC micelles. Backbone ribbon trace (A) and all-atom view (B) of the overlaid twenty lowest-energy conformers of TIRAP PBM in DPC micelles. Side chain atoms are colored in purple, whereas backbone atoms are colored in gray. (C) Surface and ribbon representation of TIRAP PBM with the side chains of the residues involved in PtdIns(4,5)P$_2$ binding. (D) Color-coded Van der Waals surface of TIRAP PBM in DPC micelles based on the electrostatic potential at the surface. (E-F) Backbone dynamics of DPC-embedded TIRAP PBM in the absence and presence of PtdIns(4,5)P$_2$. $^{15}$N, $^1$H NOEs (A) and R1 * R2 (B) relaxation parameters of individual residues of TIRAP PBM were measured in the absence (empty circles) or presence (filled circles) of 12-fold excess of PtdIns(4,5)P$_2$. Each of the plots represents the average of two independent experiments.
1.6 ORIENTATION OF TIRAP PBM IN DPC MICELLES

The NMR titration with PtdIns(4,5)P₂ reveals key interacting residues in TIRAP PBM. In addition to specific lysine residues, Leu18 and Leu30 are also perturbed upon PtdIns(4,5)P₂ binding. This led us to hypothesize that TIRAP might insert into the membrane through association of these leucine residues to fatty acid chains of PIPs in the membrane. This becomes evident as the PtdIns(4,5)P₂ headgroup, IP₃, is not sufficient as a ligand for TIRAP PBM (Fig. 5F, H). To investigate details of TIRAP PBM association with the membrane, paramagnetic studies were carried out to determine the depth of membrane insertion of TIRAP PBM using DPC micelles. Paramagnetic transition ions, such as Mn²⁺, enhance relaxation in a distance-dependent manner and, therefore, broaden NMR resonances from protein residues exposed to aqueous solvent [94]. In addition to Mn²⁺, two more paramagnetic reagents, 5-DSA and 16-DSA were used. In the case of 5-DSA, the doxyl moiety is localized close to the polar head group of the micelle near the membrane-water interface, whereas 16-DSA contains a spin label near the end of the fatty acid chain; thus, the probe will be found close to the center of the micelle.

1H-15N HSQC spectra of TIRAP PBM (0.1-0.2 mM) in d₃₀DPC micelles were collected in the absence and presence of either 16-DSA or 5-DSA (0.1-2 mM each). The reduction in density of HSQC spectra was calculated as the ratios of the peak intensities of the spectra in the absence and presence of the probe. As shown in Fig. 10A, with 0.1 mM Mn²⁺, the peak intensities of most residues reduce less than half, however, with 1 mM Mn²⁺, residues at two termini showed significant peak intensity reduction. 16-DSA
showed opposite results; significant intensities reduction were observed at the central region of TIRAP PBM, whereas the two termini were protected from line broadening (Fig. 10B), indicating that the central helix of PBM is indeed embedded into micelles, while two flexible termini are located on the surface. The 5-DSA titration of TIRAP PBM did not lead to much intensity reduction with the exception of Leu18 (data not shown), which is expected since this residue may locate at the interface of micelle. Titration of TIRAP PBM with paramagnetic agents were also conducted in the presence of PtdIns(4,5)P₂, however, no different pattern was observed compared with the spectra of

![Figure 10. Probing the insertion of TIRAP PBM in DPC micelles. Paramagnetic relaxation enhancement of the backbone amide groups of TIRAP PBM induced by Mn²⁺ (A) and 16-DSA (B) at the indicated concentrations. (C) Surface representation of TIRAP PBM with the residues showing at least 25% reduction in signal by Mn²⁺ colored in purple (solvent-exposed) and by 16-DSA colored in orange (micelle-embedded). (D) Surface representation of TIRAP PBM color-coded according to the properties of its amino acids. Blue, positively charged; red, negatively charged; yellow, hydrophobic.](image)
TIRAP PBM without PtdIns(4,5)P$_2$ (data not shown), indicating that this PIP does not contribute in TIRAP PBM’s membrane insertion.

1.7 PHOSPHORYLATION IN TIRAP PBM REDUCES ITS PHOSPHOINOSITIDE BINDING

Previous work showed that TIRAP can be phosphorylated on Thr28 by IRAKs, which leads to TIRAP intracellular degradation [19]. Negatively charged amino acids, such as aspartic acid (Asp) and glutamic acid (Glu), are often employed as pseudophosphorylated variants of protein [95], however, sterical difference with phosphorylated residues are evident, and therefore, interpretation of data using Asp and Glu as phosphomimics should be considered with caution. To investigate the role of Thr28 in TIRAP’s function, we introduced an Asp residue at position 28 in TIRAP and TIRAP PBM to mimic phosphorylation, and tested their binding to PIPs using PLOA. The results show that TIRAP T28D almost abolished PtdIns(4,5)P$_2$ and PtdIns(3)P binding in TIRAP (Fig.11B, D) and drastically reduced TIRAP PBM’s binding to PIPs (Fig. 11A, C). These inhibitions may be due to the additional negative charge in TIRAP/TIRAP PBM, or because the helical conformation in TIRAP PBM become distorted.
Figure 11. Functional and structural studies of TIRAP PBM/TIRAP phosphorylation. TIRAP PBM/TIRAP and their T28D mutant (10 μg) were incubated with immobilized PtdIns(4,5)P$_2$ (A, B) or PtdIns(3)P (C, D). E, CD spectra of TIRAP PBM and its mutants were superimposed and color-coded as shown in the inset.
1.8 PHOSPHORYLATED TIRAP PBM AND T28D PBM SHOW LESS HELICAL CONFORMATION

To determine the structural basis of inhibition of TIRAP/TIRAP PBM T28D binding to PIPs, we tested the secondary structure of TIRAP PBM T28D and the Thr28 phosphorylated TIRAP PBM (PBM-P) using CD and NMR. As shown in Fig. 11E, in the presence of DPC micelles, the CD spectrum of TIRAP PBM T28D (blue line) shows dramatic conformational changes compared with wild-type TIRAP PBM (black line), thus, T28D definitely affected the overall helical conformation in PBM. TIRAP PBM-P shows similar pattern with TIRAP PBM T28D. However, TIRAP PBM T28A (green line), as expected, did not show conformational change due to alanine has a small side nonpolar side chain, which unlikely distorts the helical conformation. These data indicate that TIRAP’s membrane localization might be impaired by repulsion triggered by the negative charge in phosphorylation site, therefore, providing a mechanism to cytosolically sequester TIRAP for its degradation in the absence of TLR activation. TIRAP PBM K16A/K31A/K32A showed inhibition to PIPs (Fig. 7), but not due to the distortion of the helical conformation of the peptide (Fig. 11E, yellow line), implicating that membrane association of TIRAP require both interactions.

![Figure 12. Orientation of Thr28 residue in TIRAP PBM](image)
A. Ribbon representation of TIRAP PBM with the side chains of the residues D23, R26, and T28 labeled.
between the central helix with membrane bilayers, and between the two positively charged termini with the negatively charged phosphoinositide head groups. The NMR structure of TIRAP PBM reveals that the side chain of Thr28 faces opposite to the two flexible termini, emphasizing its role in membrane insertion (Fig. 12).

1.9 MUTATION OF THREONINE 28 TO ASPARTIC ACID DISRUPTS TIRAP’S SUBCELLULAR LOCALIZATION

To test the functional relevance of our studies in a physiological condition, I established the subcellular localization of the defective functional versions of TIRAP generated in this thesis (PIP deficient-binding, and T28D) were investigated. HEK293 cells were transfected with a pCS2-EGFP-LIC plasmid expressing a GFP-tagged version of the indicated mutants and wild-type TIRAP proteins. Rhodamine phallodin, which specifically binds F-actin, and DAPI was used to label cytoskeleton and nuclei, respectively. As previously reported [46], TIRAP, but not the negative control pCS2 vector, resides at the plasma membrane and in intracellular vesicles (Fig. 13). Intriguingly, TIRAP T28D, unlike wild-type TIRAP, concentrates in the cytosol, and forms fiber-like patterns (Fig. 13). This might because of the mutation impaired proper folding of TIRAP, thus, lead to aggregation of the protein in the cytosol. To confirm the function of phosphomimetic of TIRAP T28D, TIRAP T28A localization was also studied. In contrast to TIRAP T28D, TIRAP T28A localization was indistinguishable to that observed for the wild-type protein (Fig. 13). This result supports the hypothesis that the negative charge introduced in PBM specifically impairs TIRAP’s membrane binding. The
PIP-binding defective version of TIRAP, TIRAP K16A/K31A/K32A also disrupts membrane localization (Fig. 13), indicating that membrane binding of TIRAP requires PtdIns(4,5)P₂. These data also shows that Lys15, which was indicated to be important for membrane localization of TIRAP [46], seems not to be critical for that function, emphasizing that the NMR titration clearly pinpointed the critical residues of TIRAP required for plasma membrane localization.

Figure 13. HEK293 subcellular localization of TIRAP, TIRAP T28D, TIRAP T28A, and TIRAP K16A/K31A/K32A. HEK293 cells were transfected with plasmids containing the cDNA that codifies for TIRAP or the indicated mutants. Green signal represents GFP conjugated to TIRAP; red signal represents phalloidin that stains actin stress fibers; blue signal shows nuclei stained with DAPI. Every cell picture shows an overlay of the signals indicated above.
CHAPTER V: DISCUSSION

TIRAP is a key innate immunity adaptor in TLR signaling. Its function in plasma membrane MyD88-dependent TLR signal transduction is well established. In order to propagate signal of the presence of the pathogen, TIRAP’s membrane targeting is essential for bridging MyD88 to membrane receptor to lead formation of myddosome. However, the structural basis and mechanism of TIRAP’s membrane association remain unclear. Recent discovery about its promiscuous binding to PtdIns(4,5)P$_2$ at the plasma membrane and PtdIns(3)P in endosomal membranes diversifies the subcellular sites of TLR signaling, which make it more urgent than usual to answer this question. Thus, I seek to understand the ligand binding properties of TIRAP and how it modulates TIRAP’s function. The biophysical basis of PIP binding by TIRAP will add to the knowledge of understanding how TIRAP localizes in different cellular compartments and the modulation of TLR signaling. The detailed molecular mechanisms of TIRAP’s membrane binding will provide insight into beneficial strategies for controlling TLR pathway and, hence, for preventing TLR-related human diseases.

In the present study, the solution NMR structure of the DPC micelles embedded TIRAP PBM was solved. TIRAP PBM is intrinsically disordered but folds into a helical secondary structure in the presence of membrane mimics. Both head group and acyl chains of the PIP are required for TIRAP PBM binding. Both positively charge (Lys16, Lys31, and Lys32) and hydrophobic (Leu18 and Leu30) key binding residues in TIRAP PBM were identified, implicating that these residues may contribute for membrane targeting of TIRAP and, consequently, the formation of a central helix in TIRAP PBM. Intriguingly, the phosphomimetic of TIRAP inhibits its binding to PIPs, more importantly,
disrupt its localization to membranes in cell-based assays (Fig. 13). The mutation of three lysine residues also impairs TIRAP’s membrane targeting (Fig. 13). Kagan and Medzhitov reported that mutation of four lysine residues (Lys15, Lys16, Lys31, and Lys32) in TIRAP results in a loss of membrane binding [46]. Based on our study, mutation of only three lysine residues is sufficient to disrupt TIRAP’s membrane localization (Fig 13). Unlike the smeared distribution in cytosol in macrophages [46], triple mutation of lysine residues in TIRAP showed an aggregated-like pattern in the cytosol of HEK293 cells.

The presence of a central helix in the structure of TIRAP PBM is a novel finding. A central helix in TIRAP PBM is formed in the presence of membrane mimics. The above talked helix may embed in micelle, whereas the two termini may localize at the surface of micelle. The central helical region (Leu18-Leu30) in TIRAP PBM is well conserved in primates (Fig. 7C), but not in mice and rat, indicate a different mechanism of TLR signaling. More importantly, at position 28, lower eukaryotes (mice and rat) have an alanine instead of threonine, suggest primates evolved to a different modulation on TIRAP’s function. The NSR data confirmed the helical conformation in TIRAP PBM, and indicate PtdIns(4,5)P₂ binding may stabilize TIRAP membrane association (Fig. 9E-F).

These data are consistent with our CD results that DPC-embedded TIRAP PBM exhibits a helical secondary structure in the presence of DPC-free PtdIns(4,5)P₂ (data now shown). The crystal structure of C-terminal TIR domain (residues, 79-221) of TIRAP was first reported at 2011 by Valkov and colleagues [48]. In the past five years, more structural properties of TIRAP TIR [96] including two SNP, TIRAP D96N and TIRAP
S180L [49] were characterized. However, structural or biophysical studies on the TIRAP PBD (or PBM) have been halted. Our CD and NMR results (data not shown) on TIRAP PBD did not show conformational change in the presence of membrane mimics. However, the PBM showed a helical secondary structure, which might be masked by additional unfolded regions in PBD. The structural properties of PBM elucidated its pivotal role in TIRAP’s localization. The lack of folded structure in the N-terminal PBD makes it evident that PBM’s structural properties are key factors for TIRAP’s membrane targeting. Knocking out of the PBM [46] or the first 29 residues (covered the central helix of PBM) [48] result in a loss of TIRAP’s proper plasma membrane localization. The phosphorylation on Thr28 distorts the helix in PBM and inhibits its binding to PIPs and further disrupt its plasma membrane association. Thus, It is proposed that the helical conformation of PBM is required for TIRAP’s proper localization, and provides an explanation how TIRAP associates with cell membranes. The central hydrophobic helix may contact with the lipid bilayer through hydrophobic interaction with acyl chains, and two flexible and hydrophilic termini of PBM with positively charged residues (lysine) interact with the negatively charged headgroup of PIPs at the membrane surface. The phosphorylation by kinases leads to disassociation of TIRAP with membrane due to the lack of the central helix. These studies provide explanation of TIRAP’s function and modulation.

Intrinsically disordered proteins/regions (IDPs/IDRs) have been characterized as highly enriched in charged and polar residues, as well as glycine and proline [97], and may fold upon binding to nature partner [98]. All these features were found in TIRAP PBM. It
has large number of lysine residues (7 out of 21), and that the electrostatic potential surface of TIRAP PBM is positively charged (Fig. 9D). The crucial role of PBM in TIRAP’s membrane targeting and TLR signaling once again challenges the traditional notion in biology that a specific function of a protein is determined by its unique 3D structure. Our finding on TIRAP PBM is consistent with recent studies on IDPs/IDRs that are frequently associated with cellular control mechanism and signaling, and have been identified at the center of “hubs” in protein interaction networks [99-102]. The intrinsically disordered structure of TIRAP N-terminal domain may also beneficial for its promiscuous lipid binding and diversified subcellular localization since IDPs/IDRs are able to interact with different partners due to their ability to adopt distinct conformation.

To conclude, this study investigated the structural properties of TIRAP N-terminal PIP binding domain, and identified an intrinsically disordered region that undergoes a partial folding-upon-binding switch in the presence of membrane mimics. The proper folding of above-mentioned region is necessary for TIRAP’s function. Our work brings a molecular insight into TIRAP’s ligand recognition and membrane targeting, and provided biophysical information and explanation on TIRAP’s modulation of membrane binding.
CHAPTER VI: SIDE PROJECTS

Part I: TOM1 NEGATIVELY MODULATES BINDING OF TOLLIP TO PHOSPHATIDYLINOSITOL 3-PHOSPHATE VIA A COUPLED FOLDING AND BINDING MECHANISM

CONTRIBUTION TO THE PROJECT

In this project, we establish that Tollip TBD undergoes a Tom1 GAT-mediated folding-upon-binding mechanism that is required to inhibit binding of Tollip to PtdIns(3)P. I contributed to characterize that Tom1 GAT simultaneously bind Tollip TBD and C2 domains, as well as that Tom1 GAT binds to PtdIns(3)P at the first and second helices. I purified Tom1 GAT, Tollip TBD, and Tollip C2, performed NMR titration experiments of $^{15}$N-Tollip TBD with Tollip C2 in the presence of Tom1 GAT.
Tom1 Modulates Binding of Tollip to Phosphatidylinositol 3-Phosphate via a Coupled Folding and Binding Mechanism

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http://dx.doi.org/10.1016/j.str.2015.07.017

SUMMARY

Early endosomes represent the first sorting station for vesicular ubiquitylated cargo. Tollip, through its C2 domain, associates with endosomal phosphatidylinositol 3-phosphate (PtdIns(3)P) and binds ubiquitylated cargo in these compartments via its C2 and CUE domains. Tom1, through its GAT domain, is recruited to endosomes by binding to the Tollip Tom1-binding domain (TBD) through an unknown mechanism. Nuclear magnetic resonance data revealed that Tollip TBD is a natively unfolded domain that partially folds at its N terminus when bound to Tom1 GAT through high-affinity hydrophobic contacts. Furthermore, this association abrogates binding of Tollip to PtdIns(3)P by additionally targeting its C2 domain. Tom1 GAT is also able to bind ubiquitin and PtdIns(3)P at overlapping sites, albeit with modest affinity. We propose that association with Tom1 favors the release of Tollip from endosomal membranes, allowing Tollip to commit to cargo trafficking.

INTRODUCTION

In higher eukaryotes, protein internalization, lipid membranes, and extracellular fluid are taken from a diverse array of endocytic pathways through processes that involve recruitment of endocytic proteins as well as changes in plasma membrane curvature. The mechanism of protein internalization (cargo) includes ubiquitylation and endocytosis, followed by cargo delivery into early endosomes (Platta and Stenmark, 2011). Cargo is further sorted into the intraluminal vesicles of late endosomes or multivesicular bodies (MVBs) for later degradation in the lysosomal lumen. Some cargo, however, is recycled back to the plasma membrane for additional rounds of ligand binding and further internalization.

The endosomal signaling output strictly depends on the presence of adaptor proteins as well as the endosomal lipid membrane composition, which is predominantly enriched with phosphatidylinositol 3-phosphate (PtdIns(3)P) at the cytosolic face (Gillooly et al., 2000). A number of peripheral cytosolic proteins are recruited to endosomal membranes by recognition of PtdIns(3)P through their phosphoinositide-binding modules, which exhibit a modest affinity for the lipid. This property allows for their rapid and reversible binding mode, a mechanism that depends not only on PtdIns(3)P levels but also on the presence of pre-localized binding partners (Cullen, 2008). Ubiquitylation of cargo serves as a sorting signal for delivery from the plasma membrane to the early endosomes. As such, cargo is recognized by adaptor proteins, which work coordinately to generate unique sorting membrane domains. For example, the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) is an FYVE domain-containing protein that binds PtdIns(3)P, favoring Hrs endosomal recruitment. Interestingly, Hrs weakly binds the ubiquitin moiety of cargo, thus requiring the presence of additional ubiquitin-binding proteins to make the endosomal sorting complex required for transport (ESCRT) (Jovic et al., 2010). ESCRT proteins, such as Hrs, are required for sorting ubiquitylated cargo into intraluminal vesicles and for intraluminal vesicle formation (Haglund and Dikic, 2012).

Tollip is an adaptor protein involved in protein sorting through association with Tom1, ubiquitin, and clathrin (Yamakami et al., 2003). Tollip is mainly localized on early endosomes, where it is required for both degradation of ubiquitin-conjugated proteins (Kato et al., 2004) and sorting of interleukin-1 receptor (IL-1R) at late endosomes (Brissoni et al., 2006). Also, Tollip serves as a negative modulator of Toll-like receptor (TLR) signaling pathways by sequestering interleukin receptor-associated kinases (IRAKs) (reviewed in Capelluto, 2012), and directly mediates polyQ protein removal through the autophagy pathway (Lu et al., 2014). This versatile function of Tollip is associated with its modular nature; it contains the Tom1-binding domain (TBD), C2, and CUE domains. Whereas the C2 domain of Tollip preferentially binds PtdIns(3)P and PtdIns(4,5)P2 (Ankem et al., 2011), which allows for the protein’s membrane targeting, both the C2 and CUE domains bind ubiquitin in a mechanism that impairs phosphoinositide binding (Mitra et al., 2013).
The subfamily of Tom1, Tom1L1, and Tom1L2 adaptor proteins presents an N-terminal VHS domain followed by a GAT domain. The GAT domain is a three-helix bundle module that interacts with both ubiquitin and the TBD region of Tollip (Katoh et al., 2004; Yamakami et al., 2003). Structural studies of the Tom1 GAT-ubiquitin complex suggest the presence of two potential ubiquitin-binding sites in the GAT domain (Akutsu et al., 2009). Tom1 interacts with clathrin, thus emphasizing its role in membrane trafficking (Yamakami et al., 2003), and exhibits a negative regulatory role by promoting lysosomal degradation of IL-1R, an event that requires Tollip (Brissoni et al., 2006). A model has been proposed in which IL-1R is shuttled from Tollip to Tom1, which in turn promotes the recruitment of the endosomal degradation machinery (Brissoni et al., 2006). Thus, Tollip- and Tom1-mediated cargo trafficking seem to act parallel to, or instead of, the ESCRT-0 complex, which also recognizes cargo during the initial step of endosomal sorting (Shields and Piper, 2011).

To unveil the regulatory mechanism underlying Tollip-Tom1 complex formation, we first structurally characterized Tollip TBD association with the Tom1 GAT domain. This high-affinity association triggered drastic conformational changes in both proteins, most notably by Tom1 GAT-induced Tollip TBD folding at its N terminus. We further demonstrated that the association of Tom1 with Tollip inhibits binding of Tollip to PtdIns(3)P, a mechanism that may facilitate Tollip’s commitment to Tom1-mediated endosomal cargo trafficking.

RESULTS

Tollip TBD Is Intrinsically Disordered and Partially Folds upon Tom1 GAT Domain Binding

It was previously shown that Tollip TBD is required for binding to Tom1 via its GAT domain (Katoh et al., 2004; Yamakami et al., 2003) (Figure 1A). The $^{1}H$-$^{15}N$ heteronuclear single-quantum coherence (HSQC) spectrum of Tollip TBD was characteristic of an unstructured protein as indicated by the narrow dispersion in the $^{1}H$ dimension (Figure 1B). Likewise, the far-UV circular dichroism (CD) spectrum of Tollip TBD displayed a minimum at 200 nm, a feature of unstructured protein spectra (Figure 1B, inset). Nonetheless, identification of the nuclear magnetic resonances (NMRs) of Tollip TBD was almost complete (94%; residues Val5, Gln29, and Gln30 were not assigned). Titration of the unlabeled Tom1 GAT domain into the $^{15}N$-labeled Tollip TBD resulted in dramatic improvement in resonance dispersion (Figure 1C). Resonance perturbations clustered in the first half of Tollip TBD including two N-terminal I strands. The largest chemical-shift perturbations were observed in residues Arg9, Val12, Ile20, Asp20, and Arg23 (Figure 1D). Interestingly, the GAT domain-perturbed region in TBD represents the most conserved among Tollip proteins (Figure S1A). The effect of the Tom1 GAT domain was specific, since the Tollip TBD HSQC spectrum was not perturbed by ubiquitin (Figure S1C), a ligand of the Tollip C2 and CUE domains (Mitra et al., 2013), nor by PtdIns(3)P (Figure S1D), a ligand of the C2 (Ankem et al., 2011) and GAT domains (see Figures S6B and S6D) (Boal et al., 2015).

Tollip TBD Is Intrinsically Disordered and Partially Folds upon Tom1 GAT Domain Binding

(A) Schematic representations of the Tollip and Tom1 domain boundaries and their ligand partners. Tollip residues F21 and K162, relevant for Tom1 GAT and PtdIns(3)P interactions, respectively, are labeled. The Tom1 N230 residue, critical for Tollip TBD binding, is also labeled.

(B) HSQC spectrum and resonance assignments of $^{15}N$-Tollip TBD. Inset: Far-UV CD spectrum of Tollip TBD. Red bar indicates the dispersion of the NMR resonances.

(C) HSQC spectrum and resonance assignments of $^{15}N$-Tollip TBD in the presence of Tom1 GAT.

(D) Chemical-shift perturbations of Tollip TBD upon Tom1 GAT domain binding. The colored dashed lines represent significant chemical-shift changes: red ($\Delta\lambda_{\text{average}} +1.5 \times SD$), orange ($\Delta\lambda_{\text{average}} +1 \times SD$), yellow ($\Delta\lambda_{\text{average}}$). Secondary structural content of Tollip TBD is displayed at the top of the histogram.

See also Figures S1 and S8.

Figure 1. Tollip TBD Is Intrinsically Disordered and Partially Folds upon Binding to the Tom1 GAT Domain

We expanded the NMR experiments to define the binding site of Tollip TBD in Tom1 GAT. The HSQC spectrum of the Tom1 GAT domain displayed scattered resonances, of which 96% were identified (residues Glu215, Ala276, Asn277, and Glu278 were not assigned) (Figure 2A). Direct comparison of the HSQC spectra of the Tollip TBD-free and Tollip TBD-bound Tom1 GAT domain revealed a drastic change in the position of the Tom1 GAT resonances (Figures 2A and 2B), suggesting that binding is accompanied by a conformational change in the protein. Most of the chemical-shift perturbations were
Intermolecular nuclear Overhauser effects (NOEs) between the Tollip TBD and Tom1 GAT domains were acquired from half-filtered NOE spectroscopy (NOESY)-HSQC experiments. Many intermolecular NOEs were detected, and the most evident included Tollip TBD Leu17 with Tom1 GAT Ala263 and Met264 (located in helix α2), Tollip TBD Gin19 with Tom1 GAT Thr260 and Cys261 (helix α2), and Tollip TBD Phe21 with Tom1 GAT Glu223, Leu224, Glu225, Met226, and Val227 (helix α1), and Met264 (helix α2). Of note, the Tollip TBD Gin19 backbone resonance could not be assigned in the Tom1 GAT-bound state. The Tollip TBD Leu17 backbone resonance was perturbed by Tom1 GAT (Figure 1C), but the Tom1 GAT-induced conformational change of Tollip TBD could mask the significance of the amino acid in Tom1 GAT binding from chemical-shift data analysis. Thus, the NOE data suggest that many of the Tom1 GAT- and Tollip TBD-mediated resonance perturbations in Tollip TBD and Tom1 GAT, respectively (Figures 1C and 1D, and 2B and 2C), are a consequence of conformational changes in the proteins.

The Tom1 GAT-Bound Tollip TBD Structure Reveals N-Terminal Folding

To gain structural insights into the Tollip-Tom1 complex, we sought to solve the solution structure of Tollip TBD in the Tom1 GAT-bound state. Initial structural analysis of Tom1 GAT-bound Tollip TBD rendered a TBD structure with residues Ala2-Ser6 and Pro11-Ile14 adopting a single anti-parallel β-sheet, whereas residues Ala37-Tyr45 formed a short C-terminal helical element that capped the β-sheet element of the protein (data not shown). However, the presence of a well-defined C-terminal helix in Tollip TBD was not in agreement with TalosN (Shen and Bax, 2013) analysis based on chemical shifts (data not shown). We therefore measured the internal backbone motions of Tom1 GAT-bound Tollip TBD by analysis of the $^{1}H$-$^{15}N$ heteronuclear NOEs and the $^{15}N$ relaxation times. The $^{1}H$-$^{15}N$ NOE profile showed that the first 22 residues of Tollip TBD were highly ordered, whereas the C-terminal half of the protein was disordered (Figure S2A). Considerable internal motion at the C terminus of Tom1 GAT domain-bound Tollip TBD was also observed when the T1 and T2 relaxation times as well as R1$^{*}$R2 were estimated (Figures S2B–S2D). Together, these data suggest that the conserved N-terminal Tollip TBD becomes ordered upon Tom1 GAT binding, whereas its C-terminal region remains relatively disordered. We speculate that the presence of a putative helix in the initial structure can represent a transient structural element in Tollip TBD. We therefore generated the Tom1 GAT-bound structure of Tollip TBD (residues 1–22), which still exhibited an anti-parallel β-sheet at the N terminus (Figure 3A and Table 1). Thus, the Tom1 GAT-induced chemical-shift perturbations in Tollip TBD could be mapped onto its structure (Figure 3B). The structure of the Tollip TBD-bound state Tom1 GAT domain (Figure 3C and Table 1) displayed a three-helix bundle, consistent with that reported in the ubiquitin-bound state (Akutsu et al., 2005), but the α3 helix was four residues shorter in the Tollip TBD-bound state. Residues of the Tom1 GAT domain, whose resonances were perturbed by Tollip TBD, were clustered on the Tom1 GAT domain structure (Figure 3D). Given the limited number of identified intermolecular NOEs (Table 1), we were unable to confidently dock Tollip TBD onto the Tom1 GAT domain.

Figure 2. Identification of the Tollip TBD-Interacting Residues in Tom1 GAT
(A and B) HSQC spectra and resonance assignments of $^{15}N$-Tom1 GAT in the absence (A) and presence (B) of Tollip TBD.
(C) Normalized chemical-shift perturbations in the backbone amides of Tom1 GAT induced by Tollip TBD. The colored dashed lines represent significant chemical-shift changes as described in the legend of Figure 1. Secondary structure of the Tom1 GAT domain is depicted at the top. See also Figures S1, S3, and S8.
structure. Nonetheless, based on Tollip TBD structural conformation and NOE data, the Tom1 GAT-binding site in Tollip TBD displays a loop with three conserved interacting residues, Leu17, Gln19, and Phe21, which form a relatively hydrophobic groove pocket that contacts the Tom1 GAT α1 and α2 helices, which are also mostly hydrophobic.

Identification of Critical Residues Involved in Tollip TBD-Tom1 GAT Association

NMR data indicated that Tollip TBD bound tightly to the Tom1 GAT domain in a slow exchange regime, since subequimolar concentrations of Tollip TBD showed two states for most of the Tom1 GAT resonances, whereas an excess of the unlabeled partner did not induce further changes in Tom1 GAT spectra (Figure S3). We therefore determined the affinity between Tom1 GAT and Tollip TBD using surface plasmon resonance (SPR) titrations. Tollip TBD bound immobilized Tom1 GAT with high association and low dissociation rates, and with an estimated dissociation constant ($K_d$) of 0.67 nM (Table 2 and Figure S4A). Additional domains in the full-length proteins may contribute to the binding, such as the Tollip C2 domain (i.e., Figure S7B), as reflected by the 8-fold increment in the conformation-associated equilibrium constant $K_{conf}$ (data not shown) that results in a 3-fold increment in affinity (Table 2 and Figure S4B). We sought to provide additional information by introducing individual mutations on surface-exposed and conserved interacting residues (Figure S1). Site-directed mutagenesis of Tollip TBD-interacting residues of Tom1 GAT such as Glu223, Val227, and Asn230 (helix α1) and Leu257, Met264, and Arg267 (helix α2) reduced their interaction with Tollip TBD (Table 2 and Figures S4C–S4H) but did not affect protein folding, as judged by comparison with the wild-type Tom1 GAT domain spectrum using CD spectroscopy (data not shown). Mutations on other residues presumably involved in Tollip TBD binding, such as Met226 (helix α1) and Cys261 (helix α2), did not drastically affect Tollip TBD binding (Table 2 and Figures S4I–S4J), indicating that these residues do not contribute to complex formation in a critical manner. Alanine mutations in Tollip TBD Arg9/Val12 and Asp20/Arg23 reduced Tom1 GAT binding (Table 2 and Figures S4K–S4L). Notably, mutation in Phe21, a conserved residue in Tollip (Figure S1A) that forms several intermolecular NOEs, and its NH backbone resonance is shifted when bound to the Tom1 GAT domain, reducing binding affinity by ~20,000-fold (Table 2 and Figure S4M). Consequently, the affinity of Tom1 GAT N230A for Tollip TBD F21A showed ~60,000-fold reduction in the equilibrium constant $K_d$ (data not shown) leading to ~95,000-fold reduction in binding (Table 2 and Figure S4N), suggesting that these residues reduce the affinity in the initial binding event, and consequently are critical for Tom1-Tollip complex formation.

To explore the consequences of disrupting Tom1-Tollip association in a more physiological setting, we introduced mutations in the GAT and TBD regions to evaluate whether disruption of their interaction influences subcellular localization of the proteins. As previously reported, Tollip localizes in early endosomes, cytosol, and, presumably, in late endosomes (Brissoni et al., 2006; Katoh et al., 2004), and Tom1 is cytosolic unless co-expressed with Tollip, in which Tom1 predominantly co-localizes with Tollip (Katoh et al., 2004) (Figures 4A–4C).
Furthermore, subcellular localization of Tollip partially overlaps with the early endosomal antigen 1 (EEA1), consistent with spatial restriction of protein complexes on endosomal membranes, which enable unique signaling responses (Platta and Stenmark, 2011). Co-transfection of Tollip with Tom1 N230A led to an increase in cytosolic localization and reduction of vesicular localization when co-expressed with Tom1 (albeit they displayed overlapping distribution) (Figure S5B). Cellular co-expression of Tollip F21A and Tom1 N230A display a similar outcome, with a significant loss of punctate localization of Tollip F21A (Figure 4E). We speculate that this result is a consequence of Tom1 association with the Tollip C2 domain (see next section). The PtdIns(3)P-binding deficient mutant Tollip K162A (Ankem et al., 2011) shows a punctate pattern but did not merge with EEA1 (Figure S5C), suggesting that Tollip K162A is being excluded from endosomal PtdIns(3)P-enriched domains and that binding to ubiquitylated cargo likely provides for endosomal localization of the protein. Moreover, this mutant retains the ability to recruit Tom1 (Figure S5D), indicating that the association of Tom1 with Tollip is independent of Tollip’s PtdIns(3)P binding.

### Tom1 Inhibits Binding of Tollip to PtdIns(3)P

Tollip is likely recruited to early endosomal compartments by association with membrane-embedded PtdIns(3)P (Brissoni et al., 2006). By using a lipid-protein overlay assay, Tollip, but not Tom1, bound PtdIns(3)P (Figures 5A and S6A). Using the same assay, but with 3-fold higher protein concentration, Tronchere and colleagues recently reported that the Tom1 GAT domain binds monophosphate phosphoinositides, although the association seems to lack specificity (Boal et al., 2015). To shed light on these conflicting data, we further explored lipid association of the Tom1 GAT domain using more highly sensitive methods such as NMR spectroscopy. Stepwise addition of PtdIns(3)P induced minor perturbations in specific resonances of the Tom1 GAT domain following a fast-exchange regime (Figure S6B). Perturbations occurred mainly at the first half of helix α1 and the C-terminal half of helix α2, and minor perturbations were found in helix α3, suggesting that the PtdIns(3)P-binding site overlaps with that of Tollip TBD (Figure S6C and S6D).
apparent K_D of the Tom1 GAT domain for PtdIns(3)P, measured by SPR, was estimated to be \( \sim 18 \) \( \mu \)M (Table 2 and Figure S4O). As Tom1 GAT binds Tollip TBD with an affinity that is \( \sim 27,000 \)fold stronger than that of PtdIns(3)P, it is unlikely that PtdIns(3)P can displace Tollip TBD for binding to the Tom1 GAT domain. Next, we investigated the functional role of Tom1 association with Tollip at the endosomal compartments. Pre-incubation of Tollip with Tom1 abolished Tollip’s PtdIns(3)P in a concentration-dependent manner (Figure 5A). PtdIns(3)P inhibition was specific for Tollip, since binding to the phosphoinositide by the Vam7p PX domain remained unchanged in the presence of Tom1 (Figure S6E). Furthermore, Tollip F21A also bound PtdIns(3)P, and pre-incubation of this mutant with Tom1 N230A did not affect lipid binding (Figure 5A). Thus, the data indicate that the interaction of Tom1 GAT with Tollip TBD is required to block the binding of Tollip to PtdIns(3)P.

Since Tollip binding to PtdIns(3)P is mediated by its C2 domain (Ankem et al., 2011), we asked whether the Tom1 GAT domain can directly bind to the Tollip C2 domain. Whereas no resonance perturbations were observed when Tollip TBD was titrated with the Tollip C2 domain in the absence of the Tom1 GAT domain (Figure S7A), the Tom1 GAT domain directly contacted the Tollip C2 domain, even in the absence of Tollip TBD (Figures 5C, S7B, and S7C), with high affinity (K_D of 187 nM; Table 2 and Figure S4P). Interestingly, Tollip C2-mediated perturbations mainly occurred at the \( \alpha_3 \) helix of the Tom1 GAT domain (Figure 5C), which leaves the possibility of Tollip TBD binding at the \( \alpha_1 \) and \( \alpha_2 \) helices (Figure 2C). Binding of Tom1 (or its GAT domain) showed minor effects on the binding of the Tollip C2 domain to PtdIns(3)P (Figure S7D), suggesting that Tollip TBD plays a major role in the inhibition. We then examined whether the Tom1 GAT domain can simultaneously bind the TBD and C2 domains. We titrated a pre-formed \( ^{15} \)N-labeled Tollip TBD–Tom1 GAT complex with Tollip C2 domain using HSQC analysis. Addition of the Tollip C2 domain led to evident line broadening, perturbations, and splitting of some Tollip TBD resonances (Figure 5D), suggesting that a TBD-GAT-C2 complex is formed. Notably, some of the Tollip C2 domain-induced resonance perturbations of the Tom1 GAT-bound Tollip TBD complex were also observed in Tom1 GAT
domain-bound Tollip TBD spectra (Figure 2A). Overall, the data indicate that association of Tom1 with Tollip is mediated by contacts with both the TBD and C2 domains in Tollip by its GAT domain, thus inhibiting the association of Tollip with endosomal PtdIns(3)P.

Lastly, we analyzed the role of ubiquitin in Tollip TBD-Tom1 GAT complex formation. Ubiquitin binds the Tom1 and GGA3 GAT domains at two potential sites, known as 1 and 2, with site 2 being the dominant ubiquitin-binding site (Akutsu et al., 2005; Bilodeau et al., 2004). Addition of ubiquitin into the 15N-labeled Tom1 GAT domain-induced perturbations over all the protein residue resonances, but the most pronounced effects were on the residues of helix 3 (Figures S8A and S8B), where site 2 is located, with an estimated $K_D$ of ~12 µM (Table 2 and Figure S4Q). Ubiquitin-induced Tom1 GAT resonance perturbations overlapped with those induced by the Tollip C2 domain (Figure S3C), but excluded the Tollip TBD-binding site (Figure S3C). Moreover, the drastic resonance perturbations induced by Tollip TBD (Figure 2B) suggested that Tom1 GAT might adopt a different conformational state, which could preclude ubiquitin binding. These observations raise the conflicting question of whether the Tom1 GAT domain is able to bind Tollip and ubiquitin simultaneously (Katoh et al., 2004; Yamakami et al., 2003). Analytical ultracentrifugation analysis showed that Tom1 GAT formed a tight complex with Tollip TBD (Figures S6C and S8E), (Table 2), the ubiquitin-binding function of Tom1 GAT is likely abrogated by Tollip binding.

DISCUSSION

In the present study, we determined the solution NMR structures of the Tollip TBD and Tom1 GAT domains in their bound states and showed that association of Tom1 with Tollip inhibits Tollip’s PtdIns(3)P binding. Tollip TBD is intrinsically disordered and undergoes a partial folding-upon-binding switch in the presence of the Tom1 GAT domain. The flexible nature of Tollip TBD allows for an unusually high-affinity hydrophobic interaction with the Tom1 GAT domain. Intriguingly, we demonstrated that the Tom1 GAT domain, in addition to ubiquitin and PtdIns(3)P, also binds to the Tollip C2 domain. Despite the fact that Tom1 GAT associates with the Tollip C2 domain with high affinity, Tollip TBD is required to interact with Tom1 GAT to abolish the binding of Tollip to the phosphoinositide. On the basis of these observations, we propose a mechanism for Tollip’s regulation and commitment to endosomal cargo trafficking (Figure 6), given that Tollip also negatively modulates IRAK-1 (Burns et al., 2000), TLR2, TLR4, and IL-1R (Zhang and Ghosh, 2002) activities. Endosomal PtdIns(3)P facilitates recruitment of adaptor proteins, which stereospecifically recognize the phosphoinositide

Figure 5. Tom1 Inhibits Binding of Tollip to PtdIns(3)P

(A) Lipid-protein overlay assay of the indicated proteins with immobilized PtdIns(3)P. GST-Tollip was pre-incubated in the absence and presence of His-Tom1 at the indicated molar ratios for 1 hr at room temperature. GST was employed as a negative control. (B) Inhibition of binding of Tollip to PtdIns(3)P was simultaneously judged by pre-incubation of an excess of either His-Tom1 or its His-GAT domain. (C) Histogram plot of resonance perturbations for 15N-Tom1 GAT as a function of residue number. The significance of the perturbations is color coded and the procedure is as indicated in Figure 1. (D) Expansions of 1H-15N HSQC spectra regions showing residue resonances of 15N-labeled Tollip TBD-Tom1 GAT (1:1.2) in the absence (red) and presence (black) of a 2-fold excess of Tollip C2 domain. The contour level of the Tollip TBD spectrum was increased 2-fold to visualize its resonances in the presence of the Tollip C2 domain. See also Figures S6, S7, and S8.

No ternary complex composed of Tollip TBD, Tom1 GAT, and ubiquitin could be detected (Figures S6F–S8H). Taken together, our data indicate that Tom1, via its GAT domain, can associate with either Tollip (through its TBD and C2 modules) or ubiquitin. As Tollip TBD and C2 bind Tom1 GAT ~18,000- and ~60-fold stronger than ubiquitin, respectively.
through their domains (Bissig and Gruenberg, 2013). PtdIns(3)P modestly contributes to the release of the intraluminal vesicle (Wolfert et al., 2009), thus placing the lipid with more relevance to protein recruitment at the endosomal surface. Other factors, such as membrane charge and curvature, also contribute to the targeting of PtdIns(3)P effectors to endosomal compartments (Schink et al., 2013). We propose that Tollip exists in equilibrium between cytosolic and endosomal compartments, in which PtdIns(3)P provides the platform for recruitment of Tollip. Tom1 binding to Tollip triggers Tollip release from PtdIns(3)P and increases the commitment of Tollip for binding to ubiquitylated cargo, which is anchored on the same membrane domains (Figure 6). The effect of Tom1 is plausible, since its affinity for Tollip is very high and, consequently, can compete efficiently with PtdIns(3)P for Tollip binding. The disordered nature of TBD facilitates Tollip’s hydrophobic interaction with helices 1 and 2 of the Tom1 GAT domain, whereas Tollip’s C2 domain likely associates with the third helix of Tom1 GAT. Furthermore, this mechanism could be enhanced by the presence of ubiquitin-conjugated cargo, since Tom1 may bind simultaneously to ubiquitin via its VHS domain (Ren and Hurley, 2010), while remaining associated with Tollip. We observed that the cytosolic accumulation of Tom1, as a consequence of its inability to bind to Tollip, promotes cytosolic accumulation of Tollip. Likewise, cytosolic localization of endosomal Endofin was observed when a Tom1-binding defective mutant of Endofin was co-expressed with Tom1 (Seet et al., 2004), suggesting that accumulation of cytosolic Tom1 influences subcellular localization of Endofin. Given that Tom1 also binds to the Tollip C2 domain, it is plausible that, in the absence of a functional TBD, Tollip is relocated cytosolically in a Tom1-dependent manner. For example, Tollip negatively modulates innate immunity responses through cytosolic interactions with IL-1R and IRAKs, which are TBD independent (Burns et al., 2000).

Intrinsically disordered proteins (IDPs) present charged and polar residues and sequence repeats, and lack a hydrophobic core at high frequency, which favors their unfolded state (Forman-Kay and Mittag, 2013). In addition, the less abundant large hydrophobic residues of IDPs are usually engaged in the recognition of binding partners, and their ability to trigger enthalpically favorable reactions supports high-affinity interactions (Forman-Kay and Mittag, 2013). All these features are found in Tollip TBD, which is unusually rich in glutamine residues and exhibits conserved Q0 repeats. Indeed, two conserved hydrophobic residues, L17 and F21, directly contact the Tom1 GAT domain. Since IDPs are able to interact with different partners due to their ability to adopt distinct conformations, it is plausible that Tollip exhibits such an array of binding partners through interactions with its TBD that are yet to be established. It is also noteworthy that three Tollip truncated isoforms have been reported, two of which show deletions in their TBDs (reviewed in Capelluto, 2012). This is of evolutionary importance because driver mutations in human diseases are observed more frequently in disordered rather than folded protein domains, as mutations in folded domains may disrupt structure and, therefore, function in these proteins (Forman-Kay and Mittag, 2013).

Tollip has recently been reported to work by binding phosphatidylinositol monophosphatases (Boal et al., 2015) and does so under conditions whereby Shigella flexneri inositol phosphate phospha- tase increases endosomal levels of PtdIns(5)P (Ramel et al., 2011). The lipid-binding function of Tom1 is particularly relevant in higher plants, since neither Tollip nor ESCRT-0 proteins are encoded by these organisms (Blanc et al., 2009; Schellmann and Pimpl, 2009). The plant Tollip homolog has recently been demonstrated to bind phospholipids (Blanc et al., 2009), and these associations localize Tollip to early endosomes (Korbe et al., 2013). Since mammalian Tollip shares common interacting partners with ESCRT-0, it is likely that Tom1 participates in cargo transport. Tollip co-immunoprecipitates with Hrs, suggesting that they form protein complexes together (Brissoni et al., 2006) in endosomal compartments (Katoh et al., 2004). ESCRT-0 is able to recruit at least eight ubiquitin molecules simultaneously (Mayers and Audhya, 2012). Tom1 is able to bind at least three ubiquitin molecules, two by its GAT domain (Akutsu et al., 2003) and one by its VHS domain (Ren and Hurley, 2010), although these multiple interactions likely occur in the absence of Tollip. In addition, Tollip binds to ubiquitin through its C2 and CUE domains (Mitra et al., 2013; Shih et al., 2003) and forms oligomers (Yamakami et al., 2003). Given that ESCRT-0, Tollip, and Tollip bind ubiquitin with modest affinity,
simultaneous binding to multiple ubiquitin molecules may increase their avidity for cargo interactions (Schink et al., 2013). Overall, the Tom1-Tollip complex formation function is in line with what has been proposed for ESCRT-0, which is able to cluster cargo to efficiently sort them for their degradation (Mayers and Audhya, 2012).

This study uncovers the negative modulation of Tom1 on Tollip PtdIns(3)P binding. Moreover, our data demonstrate that the association of Tollip with Tom1 follows a folding-upon-binding mechanism in TBD. The Tom1-Tollip complex is favored by additional contacts between their GAT and C2 domains. The presence of additional components of the Tollip-Tom1 complex and crosstalk of these proteins with members of the ESCRT family represent areas of future investigation to enable understanding of how adaptor proteins coordinate work to mediate endosomal cargo delivery.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification
Human Tom1, Tom1 GAT (residues 215–309), and Tollip TBD cDNAs (residues 1–53) were expressed as both glutathione S-transferase (GST) and His-tagged fusion proteins, human Tollip and Tom1 C2 (residues 54–182) were expressed as GST-fusion proteins, and ubiquitin was expressed as a His-tagged fusion protein. For details, see Supplemental Experimental Procedures.

NMR Titration and Structure Determination
NMR experiments were performed at 25 °C on a Bruker 600-MHz spectrometer equipped with a TBI probe (Virginia Tech), and with cold-probe-equipped Bruker 800-MHz (University of Virginia) and Varian 900-MHz (University of Colorado at Boulder) spectrometers. 1H chemical shifts were referenced using sodium 4,4-dimethyl-4-silapentane-1-sulfonate (50 μM), whereas 13C and 15N chemical shifts were referenced indirectly using frequency ratios as described by Wistart et al. (1995). Proteins for structure determination were prepared from 15N,13N-labeled Tollip TBD with unlabeled Tom1 GAT or 15N,13C-labeled Tom1 GAT with unlabeled Tollip TBD in 20 mM NaCl, 1 mM KCl, 1 mM N-acetyl-D,L-DTT, and 1 mM NaN3. Labeled protein concentration ranged between 0.8 and 1 mM, and the molecular ratio between labeled and unlabeled protein was 1:1.2. Backbone and side-chain resonances were assigned using HCNO, HNCO, HN(CA)CO, CBCA(CO)NH, NOESY-HSQC, and total correlation spectroscopy-HSQC experiments. Intramolecular and intermolecular NOES were obtained by 15N-edited and 13C,15N-filtered NOESY-HSQC, respectively. The NOEs identified in the complex include Tollip TBD Leu17 HD1-Tom1 GAT Val162 H3; Tollip TBD Leu17 HD2-Tom1 GAT Val162 H3; Tollip TBD Leu17 HD1-Tom1 GAT Met264 H3; Tollip TBD Leu17 HD2-Tom1 GAT Met264 H3; Tollip TBD Gin19 HD1-Tom1 GAT Thr268 H3; Tollip TBD Gin19 HD2-Tom1 GAT Thr268 H3; Tollip TBD Gin19 HD1-Tom1 GAT Cys261 H3; Tollip TBD Phe21 H3; and Tollip TBD Phe21 H3. 1H,15N NOES were measured by comparing intensities of 1H-15N relaxation rates and 1H,13C NOE intensities with either 5 s of 1H saturation or a 5-s delay preceding 1H saturation.

SPR Kinetic Studies
SPR measurements were performed at room temperature using a BIAcore X-100 instrument (GE Healthcare). Protein-protein interaction experiments were performed in 10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.005% P20 (running buffer). A purified His-tagged protein (ligand) (2–10 nM) was immobilized on a nitrilotriacetic acid (NTA) sensor chip (–15–110 response units) according to the manufacturer’s instructions. The NTA chip was saturated with 0.5 mM NCl3 in each cycle, then the protein analyte was diluted into the running buffer at different concentrations and injected over both ligand and reference flow cells at a flow rate of 30 μl/min. The signal from the experimental flow cell was collected by subtraction from the reference flow cell. The chip was regenerated with 10 mM HEPES (pH 8.3), 150 mM NaCl, 3 mM EDTA, and 0.005% P20 before the next cycle. Association of Tom1 GAT to PtdIns(3)P-containing liposomes was monitored using an L1 sensor chip, similar to what we have previously described (Krickem et al., 2011). Apparent Kd values were calculated using the BIACalculator software, version 2.0 (GE Healthcare), and in all cases, data best fit the two-state conformational change model. In this model, an initial binding event takes place, which is measured by the equilibrium association constant Ka (Kd = 1/KA). Furthermore, the protein(s) undergoes a reversible conformational change in the bound state, which is calculated by the equilibrium constant Kcord = ka/kd and Kd = Ka1 + Kcord (De Mol and Fisher, 2008).

Cell Cultures and Immunofluorescence Analysis
HeLa cells (American Type Culture Collection) were grown in Dulbecco’s minimal essential medium supplemented with 10% (v/v) heat-inactivated fetal BSA (Sigma) in a 37 °C humidified incubator in the presence of 5% CO2. Cells were split every 2–3 days and maintained at 50% confluence. Transient transfection of Tom1 in pCS2+– Flag and Tollip (pEGFP-C1) plasmids was performed using Lipofectamine LTX (Invitrogen) using 1 μg of DNA and 2.2 μl of Lipofectamine in OptiMEM reduced medium (Invitrogen) for 10 cells. Cells grown on glass coverslips were fixed and permeabilized in 3% formaldehyde, 0.1% Triton X-100, and PBS for 7 min at room temperature, and blocked with 20% goat serum in 0.1% Triton X-100 and PBS. Primary antibody (mouse anti–Flag M2 [Sigma] or mouse anti-EEA1 [BD Transduction Laboratories] antibodies) was added to cells and incubated at 4 °C overnight followed by incubation with Cy3-conjugated goat anti-mouse antibody.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and eight figures and can be found with this article online at http://dx.doi.org/10.1016/j.str.2015.07.017.

AUTHOR CONTRIBUTIONS
D.G.S.C. designed the project and wrote the manuscript with input from all authors. S.X. purified proteins, and collected and processed most of the SPR experiments. X.Z. and M.K.B. cloned and generated constructs. C.V.F. designed and interpreted immunofluorescence procedures. D.G.S.C., J.H.B., J.F.E., G.S.A., and X.Z. collected and processed NMR data. S.X. and M.K.B. cloned and generated constructs. C.V.F. designed and interpreted immunofluorescence procedures. M.K.B. purified proteins and carried out the immunofluorescence procedures under C.V.F.'s guidance. K.I.F. purified proteins and carried out lipid-protein overlay assays.

ACKNOWLEDGMENTS
We thank Dr. Janet Webster for assistance during preparation of the manuscript. This work was funded by the American Heart Association Grant in-aid (13GRNT16960080) and by the Thomas F. and Kate Miller Jeffress Memorial Trust (J1028) to D.G.S.C., the Virginia Tech SURF fellowship, the Atlantic Coast Conference Creativity and Innovation, and the Sigma Xi Virginia Tech chapter grants to K.I.F. Some of the NMR data presented in this work was collected using NMR equipment purchased with an NIH High End Instrumentation grant (S10 RR023035) and housed in the Biomolecular Magnetic Resonance Facility at the University of Virginia.

Received: June 13, 2015
Revised: July 24, 2015
Accepted: July 28, 2015
Published: August 27, 2015

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Part II: BIOPHYSICAL AND MOLECULAR DYNAMICS STUDIES OF PHOSPHATIDIC ACID BINDING BY THE DVL-2 DEP DOMAIN

CONTRIBUTION TO THE PROJECT

In this project, we structurally defined the phosphatidic acid (PA)-binding properties of the Dvl2 DEP domain. I contributed to determine the binding affinity between Dvl2 DEP and PA using tryptophan fluorescence. I also characterized the secondary structural properties of Dvl2 DEP domain and several key mutants. I purified Dvl2 DEP protein, and conducted CD and tryptophan fluorescence experiments. I also deposited resonance of assignments of Dvl2 DEP (Entry 19584) at the Biological Magnetic Resonance Data Bank.
Biophysical and Molecular-Dynamics Studies of Phosphatidic Acid Binding by the Dvl2 DEP Domain

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ABSTRACT The Wnt-dependent, β-catenin-independent pathway modulates cell movement and behavior. A downstream regulator of this signaling pathway is Dishevelled (Dvl), which, among other multiple interactions, binds to the Frizzled receptor and the plasma membrane via phosphatidic acid (PA) in a mechanism proposed to be pH-dependent. While the Dvl DEP domain is central to the β-catenin-independent Wnt signaling function, the mechanism underlying its physical interaction with the membrane remains elusive. In this report, we elucidate the structural and functional basis of PA association to the Dvl2 DEP domain. Nuclear magnetic resonance, molecular-dynamics simulations, and mutagenesis data indicated that the domain interacted with the phospholipid through the basic helix 3 and a contiguous loop with moderate affinity. The association suggested that PA binding promoted local conformational changes in helix 2 and β-strand 4, both of which are compromised to maintain a stable hydrophobic core in the DEP domain. We also show that the Dvl2 DEP domain bound PA in a pH-dependent manner in a mechanism that resembles deprotonation of PA. Collectively, our results structurally define the PA-binding properties of the Dvl2 DEP domain, which can be exploited for the investigation of binding mechanisms of other DEP domain-interacting proteins.

INTRODUCTION

Proteins interact with membranes, and depending on the mode of their association, are usually classified as peripheral or integral. Peripheral proteins contact membranes through different surface moieties, whereas integral proteins insert into lipid bilayers. A well-characterized peripheral protein is Dishevelled (Dvl), which is the branch point of the canonical Wnt/β-catenin signaling pathway. It regulates cell-fate specification and proliferation, and participates in the noncanonical Wnt/planar cell polarity (PCP) pathway to control cell polarization, among other functions (1). Dysregulation of Wnt signaling is often associated with human birth defects, cancer, neurodegeneration, and osteoporosis (2,3). Whereas Wnt proteins trigger Dvl phosphorylation (4) and Dvl-dependent signalosome formation at the boundaries of the plasma membrane in the canonical Wnt signaling pathway (5), an asymmetric membrane association of Dvl is observed in the Wnt/PCP signaling branch (6). However, it is unknown whether Dvl employs the same mechanism of membrane targeting in both pathways. In the Wnt/β-catenin signaling pathway, the absence of Wnt facilitates the formation of a destruction complex that leads to cytosolic β-catenin degradation. Binding of a specific Wnt protein to both the Frizzled (Fz) receptor and LDL-related protein 5/6 coreceptor complex promotes Dvl recruitment to the plasma membrane in complex with axin, a scaffolding protein required for formation of the β-catenin destruction complex. Axin sequestration by Dvl results in dissociation of the β-catenin destruction complex, resulting in β-catenin accumulation and nuclear translocation, which facilitates Wnt-responsive gene transcription. The role of Dvl in noncanonical Wnt pathways is less clear. The PCP pathway relies on formation of the protein complexes that are asymmetrically distributed in the cell. During Drosophila wing cell polarization, Dvl accumulates distally in complex with other PCP proteins but is rarely found proximally (7).

Dvl proteins are multifunctional scaffold proteins with three highly related isoforms in mammals (Dvl1, 2, and 3) but with only one form found in Drosophila (8). The expression patterns of these isoforms overlap significantly during mouse development, suggesting that they have redundant functions, although unique functional regions have been identified in Dvl3 (9). Dvl proteins consist of several modules, including DIX (Dishevelled and axin), PDZ (postsynaptic density 95, disk large, and zonula occludens-1), and DEP (Dishevelled, Egl-10, and pleckstrin) domains. All three Dvl domains are critical in the canonical Wnt pathway (10). Both PDZ and DEP, but not DIX, are necessary for function of the PCP pathway (10). The DIX domain is involved in the formation of Dvl dynamic polymers, axin, actin, and vesicular association (11–13), whereas the PDZ domain binds to the Fz receptor, which is necessary to amplify Wnt signaling to downstream effectors (14). The DEP domain is a conserved module also found in Egl-10, pleckstrin, and within the regulator of the G protein signal family including Epac2, the R7 subfamily, and the yeast Sst2 proteins (15). The C-terminal DEP domain is involved in Dvl membrane targeting and Frizzled interactions (16).
The tertiary structure of several DEP domains displays a common α/β fold with some differences in the number of α/β elements (17–19). The presence of a cluster of basic residues on the DEP domain surface is suggested to be critical for Dvl membrane targeting (17). More recently, Simons et al. (20) initially characterized Dvl1 DEP domain association to the plasma membrane and proposed a mechanism in which basic residues, located in helix 3, would preferentially bind phosphatidic acid (PA). As a result, the DEP-PA association has been proposed to depend on intracellular pH, as demonstrated by the role of the Na+/H+ exchanger Nhe2 studies (20).

To obtain further insights into the membrane-binding properties of Dvl, we carried out structural, biochemical, mutagenesis, and molecular-dynamics simulation studies of the interaction of the Dvl2 DEP domain with PA. By obtaining the backbone resonance assignments of the Dvl2 DEP domain and monitoring the nuclear magnetic resonance (NMR) chemical shift changes of the protein induced by PA, we experimentally identified the amino acids directly and indirectly associated with lipid binding. Whereas helix 3 and a conserved loop region between β-strand elements 3 and 4 are compromised in PA ligation, conformational changes in the protein could occur in helix 2 and β-strand 4. Our results also indicate that PA recognition by Dvl2 DEP domain is pH-dependent and occurs with modest affinity, emphasizing that membrane targeting of Dvl should be facilitated by additional interactions of the domain with Fz at nonoverlapping sites.

MATERIALS AND METHODS

NMR spectroscopy

NMR samples contained 0.1–1 mM of uniformly 15N and 13C,15N Dvl2 DEP domain, 90% H2O/10% 2H2O, 20 mM d6-sodium citrate (pH 5.5–7.5) buffer, 100 mM NaCl, 1 mM DTT, and 1 mM NaN3. Lipid binding was monitored after chemical shift perturbations in the 1H,13N heteronuclear single quantum coherence (HSQC) spectra of 100-μM Dvl2 DEP domain after addition of 16-fold excess of dihexanoyl PA (DHPA; Avanti Lipids, Alabaster, AL), and was acquired at 25°C, following the addition of 100 mM NaCl, giving a final system containing 1:16 protein/lipid. Lipid samples contained 0.1–1 mM of uniformly 13C,15N-labeled POPA. Parameters for DHPA were taken from the headgroups and first six carbons of the palmitoyl oleoyl PA (POPA) acyl chains (34), substituting 13C on INOVA 600 and 500 MHz spectrometers (Varian, Cary, NC) equipped with triple-resonance shielded probes with z-axis pulse field gradients. The pH of the protein sample was confirmed and adjusted if needed before each run. Chemical shift perturbations were calculated according to the following formula (21):

$$\Delta \delta(1H, 15N) = \left[ (\Delta \delta(1H))^2 + (\Delta \delta(15N))^2 / 2 \right]^{0.5}.$$  

Triple-resonance experiments of 13N,15N Dvl2 DEP domain (1 mM) were performed at 25°C on INOVA 600 and 500 MHz spectrometers (Varian, Cary, NC) equipped with triple-resonance shielded probes with z-axis pulse field gradients. 1H chemical shifts were referenced using sodium 4,4-dimethyl-1-silapentane-1-sulfonate (50 μM) as an internal reference. Sequential assignments of the backbone 1H, 13C, and 15N resonances were made from 1H,13N-HSQC, CBCA(CO)NH, HNCACB, HNCO, and H(CCO)NH two-dimensional NMR spectroscopy experiments (τmix = 50 and 135 ms) (22–24). Spectra were processed with the software NMRPIPE (National Institutes of Health, Bethesda, MD) (25) and analyzed using the softwares PIPPI (National Institutes of Health, Bethesda, MD) (26) and NMRDRAW (National Institutes of Health, Bethesda, MD). The resonance assignments of the Dvl2 DEP domain have been deposited in the Biological Magnetic Resonance Data Bank under accession No. 19584.

Molecular-dynamics simulations

The mouse Dvl2 DEP domain structure was generated by homology modeling based on its sequence identity (69.7%) to mouse Dvl1 DEP (PDB:1FSH; residues 404–502) using AL2TS (http://proteinmodel.org/AS2TS/AL2TS/al2ts.html) and validated using the SWISSMODEL workserver (http://swissmodel.expasy.org/). The stability of the Dvl2 DEP domain structure over time was judged by its root-mean-square deviation and root-mean-square fluctuation parameters (see Fig. S1 in the Supporting Material). All simulation preparation steps and analyses were conducted using the GROMACS software package, Vers. 3.3.3 (http://www.gromacs.org/) (27). All elements of the system were described by the GROMOS96 43A1 force field (28). Periodic boundary conditions were applied in all directions. The van der Waals interactions were truncated at 0.8 nm, with dispersion correction applied to energy and pressure terms to account for truncation. Nonbonded interactions were calculated using a twin-range scheme, updating the neighbor list between 0.9 and 1.4 nm every five simulation steps. The long-range electrostatics was calculated with the smooth particle-mesh Ewald method (29,30), using fourth-order spline interpolation and a Fourier grid spacing of 0.12 nm. The real-space contribution to particle-mesh Ewald was truncated at 0.9 nm. Simulations were run using a leapfrog integrator with an integration time step of 2 fs. All bonds within protein and lipid molecules were constrained using the LINCS algorithm (31) and water molecules were kept rigid using the SETTLE algorithm (32). All systems were energy-minimized using the steepest-descent method.

For each simulation, three independent trajectories were produced by generating different random velocities at the outset of equilibration, which was carried out in two phases. The initial phase employed a canonical (NVT) ensemble for 100 ps. Temperature was regulated using the Berendsen weak coupling method (33). Isothermal-isobaric (NPT) equilibration was then carried out for an additional 100 ps, using the Berendsen weak coupling method to regulate pressure at 1.0 bar. During equilibration, position restraints were placed on the heavy atoms of the DEP domain. Production simulations were carried out for 20 ns in the absence of any restraints, using the same ensemble as in the NPT stage. In the case of simulations in the presence of DHPA, the DEP domain was centered in a dodecahedral simulation box with four DHPA molecules randomly distributed around the protein in three different configurations. This setup suitably matches the molar ratio of protein/lipid used in the NMR experiments while avoiding any concentration-dependent artifacts that would arise from using a 1:16 protein/lipid.

The box was filled with simple point-charge water (34) and 100 mM NaCl. Parameters for DHPA were taken from the headgroups and first six carbons of the palmitoyl oleoyl PA (POPA) acyl chains (35), substituting a methyl group instead of a methylene for the sixth carbon in each acyl chain. Equilibration and simulation parameters are described above, with simulations conducted at 25°C, corresponding to experimental conditions. The configuration for the POPA membrane was taken from simulations conducted by Dickey and Fuller (35), applying the same force-field parameters used in that work. The charge assigned to each lipid headgroup was −1. The system was then solvated with simple point-charge water (34), followed by the addition of 100 mM NaCl, giving a final system containing ~29,000 atoms. The system temperature was set at 37°C, above the phase-transition temperature of POPA (35). Pressure was regulated semisotropically using the Berendsen method with a 2.0-ps coupling constant, allowing the membrane to deform independently in the x-y and z dimensions. The electrostatic potential surfaces were calculated using the software DELPHI (http://compbio.clemson.edu/delphi.php) (36).
RESULTS
Backbone assignments of the Dvl2 DEP domain
To structurally characterize the Dvl2 DEP domain interaction with PA, we assigned the NMR backbone resonances of the protein. The backbone amide resonances in the $^1$H,$^{15}$N HSQC spectra as well as the $\alpha$- and $\beta$-carbons were sequentially assigned to all non-proline residues based on an analysis of the correlations observed in CBCA(CO)NH and HNCACB spectra. The backbone carbonyl resonance assignments were obtained from the HNCO and HN(CA)CO experiments, and the assignments for the $\alpha$- and $\beta$-protons were obtained from the HBHA(CO)NH experiment. Ninety-five percent of $^1$H and $^{15}$N resonances of 95 backbone amides (excluding the three Pro residues) ($\text{Fig. 1}$) and $^{13}$C resonances of 92 of the 99 backbone carbonyls of the Dvl2 DEP were assigned. NMR signals from four residues (Ile$^{449}$, Glu$^{471}$, Ser$^{498}$, and Glu$^{499}$) could not be assigned. In addition, 96% of $^{13}$Ca and 97% of $^{13}$C$\beta$ resonances were assigned. The sequential connectivity of $^{13}$Ca and $^{13}$C$\beta$ carbons of a representative region of the Dvl2 DEP domain is depicted in $\text{Fig. S2}$. 

Structural analysis of Dvl2 DEP domain interaction with PA
Previous work, using a site-directed mutagenesis approach, demonstrates that the Dvl1 DEP domain binds acidic phospholipids with a preference for PA (20). However, the mutagenesis design is based on the exposed positively charged residues in the protein without any evidence of a direct role of these amino acids in lipid recognition. To precisely map the interaction surface between the Dvl2 DEP domain and PA, we titrated water-soluble DHPA into the $^{15}$N-labeled Dvl2 DEP domain and monitored chemical shift perturbations in $^1$H-$^{15}$N HSQC spectra. Several chemical shift changes and line-broadenings were observed at saturating concentrations of DHPA ($\text{Fig. 2, A and B}$).

Two distinct regions, located at the second helix (residues 461–469) and the fourth $\beta$-strand (residues 501–504) of the Dvl2 DEP were perturbed by DHPA ($\text{Fig. 2 B}$). Additional backbone perturbations were observed in the DEP N-terminal residues Ser$^{418}$ and His$^{426}$ as well as in resonances corresponding to His$^{490}$ and Lys$^{494}$, which are located between $\beta$-strand 3 and 4 near the C-terminus of the protein. Also, severe line-broadening of NMR signals were found in residues Asp$^{422}$, Leu$^{445}$, Met$^{448}$, Thr$^{491}$, Val$^{492}$, and Lys$^{494}$. By mapping the resonance perturbations on the structure of the Dvl2 DEP domain ($\text{Fig. 2 C}$), we concluded that two distinct regions on opposite sides of the protein may be involved in PA binding and/or they underwent a conformational change upon ligand association. The Dvl2 DEP domain bound to PA in a fast exchange regime on the NMR timescale, indicating that the lipid was weakly bound. DHPA-induced local conformational changes of the Dvl2 DEP domain was followed by monitoring the intrinsic fluorescence of two conserved Trp residues in the protein (Trp$^{444}$ and Trp$^{461}$; see $\text{Fig. S3}$, purple boxes). Whereas Trp$^{461}$ is located in helix 2, Trp$^{444}$ is found between $\beta$-strands 1 and 2. Interestingly, chemical shift perturbation analysis indicated that the backbone amide resonance of Trp$^{461}$ shifted in the presence of DHPA ($\text{Fig. 2 A}$). The observed Dvl2 DEP fluorescence emission trace between 310 and 410 nm was broad, likely due to the presence of Trp$^{444}$ and Trp$^{461}$.
in distinct microenvironments (Fig. 3). Fluorescence emission of Dvl2 DEP domain was quenched by DHPA, suggesting solvent accessibility of the Trp residue/s of the protein upon DHPA binding, and saturated at 32-fold excess of the ligand (Fig. 3) with a $K_D$ for DHPA of 4.30 ± 0.66 μM ($\chi^2 = 0.00009$).

Far-UV circular dichroism (CD) analysis of the Dvl2 DEP indicates that the protein exhibited a characteristic spectrum of an $\alpha/\beta$ protein (see Fig. S4 A). Addition of DHPA did not induce major changes in the far-UV spectrum of the protein (see Fig. S4 A). Although the far-UV CD results show that no significant changes in secondary structure are present, evaluation of the near-UV CD spectra was carried out to confirm that the reduction of fluorescence intensity observed upon DHPA binding is indeed reflecting conformational changes in the Dvl2 DEP domain. The near-UV CD spectrum, which indicates the presence of tertiary structure in the protein, exhibited significant optical activity arising from the four Tyr and two Trp residues of the DEP domain in the 275–300 nm region and a positive signal at 260 nm, probably associated with the four Phe residues present in the DEP domain (see Fig. S4 B). A reduction of the amplitude of the CD signal is observed in the 255–265 and 290–300 nm regions when the protein is incubated with DHPA, indicating that the orientation of aromatic groups is perturbed in the DEP domain by the presence of the lipid. Overall, these observations, together with the comparison of $^{15}$N, $^1$H HSQC, and intrinsic fluorescence of the free and PA-bound Dvl2 DEP domains, suggest that the protein undergoes a local conformational change upon DHPA binding rather than a major modification of the overall protein architecture.

Dvl2 DEP domain interaction with PA using molecular-dynamics simulations

In all simulations of the DEP domain in the presence of water-soluble DHPA, the lipid molecules are bound to the DEP domain within the first 10 ns of simulation time, after which their positions remained very stable. The final configurations of each of the three simulations are shown in Fig. 4 and details of such interactions are shown in Fig. S5,
FIGURE 4 Interactions between Dvl2 DEP domain and DHPA for (A) simulation 1, (B) simulation 2, and (C) simulation 3. Residues that are responsible for binding DHPA lipids (labeled, displayed as sticks, and colored by element: C, gray; O, red; P, gold; H, white). The DEP domain is shown as a cartoon structure (and colored as a rainbow gradient from blue, N-terminus to red, C-terminus). (Blue, green, and yellow) Helices H1, H2, and H3 in the DEP domain, respectively. To see this figure in color, go online.

Identification of critical residues in Dvl2 DEP engaged in PA binding

To distinguish between key Dvl2 DEP domain PA-interacting residues and conformational changes in the protein induced by the phospholipid, we carried out mutagenesis on residues that exhibited DHPA-mediated NMR chemical shift perturbations as well as on those residues that showed interactions with the lipid from molecular-dynamics simulations. We used two different lipid-binding assays. Mutations in residues His464, His465, and Phe469 did not significantly affect binding (Fig. 6 A), suggesting that local conformational changes, rather than direct interaction with the lipid, occur in and around the second helix of the protein. Mutations on the DEP domain residues His490 and Lys494, which induced NMR chemical shift perturbations (Fig. 2) and interacted with DHPA in our simulation studies (Fig. 4), severely reduced PA binding (Fig. 6 A). We were unable to evaluate additional putative PA-interacting Dvl2 DEP domain residues, as suggested from NMR titration experiments, because alanine mutation of His420 and Lys462 and
serine mutation of Cys$^{501}$ produced misfolded or degraded products, as determined by CD spectroscopy and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses (data not shown).

Studies on the Dvl1 DEP domain have shown that glutamic acid mutations of residues Lys$^{408}$, Lys$^{458}$, Lys$^{465}$, Lys$^{472}$, and Lys$^{482}$, which are equivalent to Dvl2 His$^{420}$, Pro$^{470}$, Lys$^{477}$, Lys$^{484}$, and Lys$^{494}$, respectively, abolish PA binding (20). Only resonances from the backbone amide groups of His$^{420}$ and Lys$^{494}$ (prolines are not visualized in HSQC spectra) were significantly perturbed by DHPA in Dvl2 DEP (Fig. 2). We also observed very minor perturbations of resonances mapping in helix 3, but they were not significant when compared with perturbations observed in helix 2 and β-strand 4 (Fig. 2 B). An alanine mutation of Lys$^{477}$ (helix 3) reduced PA binding (Fig. 6), which was
consistent with our simulation studies (Fig. 4), indicating that the side chain, but not the backbone of the amino acid, may play an important role in the association. Moreover, alanine mutagenesis on Arg^476 (which is also located in helix 3) abrogated lipid binding, supporting our simulation studies. Far-UV CD spectra of the most critical PA-binding mutants, such as K477A, and K494A, exhibited similar traces compared with the wild-type protein, indicating that mutations did not significantly perturb the global fold of the protein in these regions (see Fig. S9). As summarized in Table S4, our results identified the role of the third helix and a loop of the Dvl2 DEP domain, located between the third and fourth β-strand elements, in PA recognition.

**Dvl2 DEP domain binding to PA is pH-dependent**

Recent work by Simons et al. (20) proposes that Dvl recruitment to the membrane depends on pH-sensing by PA to regulate cell polarity in Drosophila. To biochemically determine the effect of the physiological pH range on the Dvl2 DEP domain binding of PA, we evaluated the association at pH values from 6.5 to 8.0. We found that the Dvl2 DEP dramatically decreased binding to PA at pH values below 8.0 (Fig. 7A), whereas the Vam7p PX domain binding to PtdIns(3)P (phosphatidylinositol 3-phosphate) was not affected, consistent with previous observations (37). To quantify the pH effect using a membrane mimic that closely resembles a lipid bilayer, a liposome-binding assay was performed in a range of physiologically relevant pH values. The DEP domain was incubated with large unilamellar vesicles, without and with DOPA, at pH values of 6.25, 6.5, 6.8, 7.1, and 7.5 and unbound (supernatant) and liposome-bound Dvl2 DEP domain (pellet) fractions were obtained by centrifugation.

By increasing the pH from 6.25 to 7.5, the Dvl2 DEP domain increased its interaction with DOPA-containing liposomes by >50% (Fig. 7B). This is in agreement with the observed defective Drosophila Dvl recruitment to the plasma membrane when the intracellular pH was dropped to pH 7.1 (20). A near-UV CD spectrum of Dvl2 DEP domain exhibited differences in their traces at pH values of 6.25 and 7.5 (see Fig. S10), suggesting that there are pH-dependent conformational changes around aromatic groups of the Dvl2 DEP domain. To better visualize this effect, we analyzed pH-dependent changes in the protein by collecting HSQC spectra at pH values of 5.5 and 7.5. Interestingly, and in agreement with the electrostatic/hydrogen-bond switch model (38), we observed that increasing the pH from 5.5 to 7.5 induced chemical shift perturbations in the Dvl2 DEP domain spectrum (Fig. 7C) which, in most cases, mirrored the perturbations observed by the addition of DHPA (Fig. 2A).

**DISCUSSION**

In this report, we precisely characterize the phospholipid binding properties of the Dvl2 DEP domain. A significant impact of the biophysical approach reported here is that it provides direct evidence for the DEP domain residues engaged in PA binding and it does so in a pH-dependent manner. As depicted in Fig. 8, the positive electrostatic potential of the Dvl2 DEP domain is distributed all over its surface at pH 5.5, whereas the positive charge is clearly localized on the PA-interacting regions, the helix 3 and a downstream loop, surrounded by negative charges at pH 7.5. This observation suggests that the DEP domain becomes more specific for its lipid ligand and that lipid binding is favored by electrostatic interactions with both helix 3 and its contiguous loop at higher pH values. The
The three-dimensional structure of Dvl1 DEP domain has been solved by NMR spectroscopy (17). The structure reveals the presence of a three-helix bundle, a $\beta$-hairpin arm, and two short $\beta$-strand elements at the C-terminus of the domain. An almost identical structure has been determined for the Dvl2 DEP domain as a chimera in complex with the $\mu_2$ subunit of the AP-2 clathrin adaptor (39).

The major feature of the DEP domain structure is the presence of a hydrophobic core formed by the three $\alpha$-helices, which maintain a stable tertiary structure. Based on the structure, a patch of basic residues in helix 3 of the Dvl1 DEP domain surface has been suggested to interact with acidic phospholipids on the plasma membrane (20). Basic residues also drive Epac1 DEP domains and Sst2 DEP interactions with phosphatidylserine, phosphatidylinositol 4,5-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate (40,41).

In addition to the loop residues His$^{490}$ and Lys$^{494}$, we observed NMR chemical shift perturbations of the Dvl2 DEP domain in backbone residues located at the helix 2 and $\beta$-strand 4 elements. Due to strong hydrophobic contacts of helix 2 with the functional helix 3, as well as the capping function of $\beta$-strand 4 on the hydrophobic core of the DEP domain (17), we therefore propose that lipid contact to the side chains of residues of helix 3 triggers both local pH and conformational changes in helix 2 and $\beta$-strand 4, which are

**FIGURE 7** Binding of the Dvl2 DEP domain is pH-dependent. (A) Lipid-protein overlay assay of DPPA binding of Dvl2 DEP domain (top) and PtdIns(3)P binding of Vam7p PX domain (bottom) at the indicated pH values. (B) Representative gels of liposome pellets showing binding of Dvl2 DEP to DOPA-containing liposomes (top) and DOPA-free liposomes (bottom) at the indicated pH values. (C) $^{15}$N-HSQC spectra of the Dvl2 DEP domain at pH 5.5 (red) and 7.5 (black). To see this figure in color, go online.

**FIGURE 8** (A and B) Electrostatic potential surfaces for the DEP domain at pH 5.5 (top) and 7.5 (bottom). The corresponding electrostatic potential surfaces are colored as gradients (red, negative; blue, positive). (Boxed) Positive charge generated on the PA-interacting regions of the DEP domain at pH 7.5. To see this figure in color, go online.
committed to maintaining the rigid hydrophobic core of the protein.

The DEP domain is required for Dvl recruitment to the plasma membrane during PCP- and β-catenin-mediated signaling (16,20). In addition, the Dvl DEP domain and a C-terminal region of the protein are responsible for the interaction with the Fz receptor to activate β-catenin-mediated signaling (16). Three motifs of Fz, two located in the third intracellular loop and one at the C-terminus of the protein, are required for binding to the Dvl central and C-terminal regions and trigger Wnt-induced β-catenin stabilization (14,16). Specifically, the Dvl1 PDZ (14) and DEP (16) domains preferentially bind to the Fz C-terminal region that contains the KTxxxW motif with $K_D$ values of 10 and 22–30 μM, respectively, and that the Dvl1 residues Lys$^{438}$, Asp$^{440}$, and Asp$^{442}$ (equivalent to Dvl2 Lys$^{446}$, Asp$^{448}$, and Asp$^{449}$, respectively) are required for this association (16). Interestingly, NMR resonances of these key Fz-interacting residues in Dvl2 are not perturbed by DHPA (Fig. 2), which makes them available for simultaneous binding with Fz and phospholipids (16). Likewise, structural analysis of the Dvl2 DEP-μ2 subunit of the AP-2 clathrin adaptor reveals that PA-independent DEP domain residues Asp$^{441}$, Lys$^{446}$, Asn$^{451}$, and Arg$^{455}$ are compromised in μ2 subunit binding (39).

PA is a relatively abundant phospholipid (~1% of total cellular lipid (42)) that exhibits a cone-shaped structure, a conformation that prevents tight packing of the small headgroup of the phospholipid with the headgroups of other lipids found at the plasma membrane (43,44). PA-binding proteins lack the presence of a conserved PA-binding site, but instead interact with the phospholipid through nonspecific electrostatic interactions between patches of positively charged amino acids and the negatively charged headgroup of PA, whereas hydrophobic amino acids may contribute to membrane insertion (45). Indeed, basic residues such as lysine and arginine are hydrogen-bond donors because of the presence of primary amines in their side chains. Consequently, Kooijman et al. (38) proposed a mechanism by which PA is recognized by PA-binding proteins known as the electrostatic/hydrogen-bond switch mechanism. In this model, proteins initially recognize the single protonated form of PA, and upon recognition, the basic amino acids of the protein can form a hydrogen bond with the PA phosphomonoester headgroup, a reaction that triggers dissociation of a proton from PA leaving the phospholipid with a 2$^+$ charge. A more negative form of PA facilitates electrostatic interactions with the protein, thus, making the protein-lipid complex more stable.

Our results demonstrate that the Dvl2 DEP domain third helix and a loop downstream of helix 3 are involved in PA binding. These regions are basic in nature, supporting the idea that the interaction of the protein with the lipid is mainly electrostatic. Biophysical data showed that two basic residues located in the loop, His$^{496}$ and Lys$^{494}$, are relevant for the interaction. We were unable to observe perturbations in the HN backbone of the residues comprising helix 3, but both mutagenesis and molecular-dynamics simulations studies demonstrated the role of this region in PA binding, emphasizing the role of the side chains of the lipid-interacting residues. Binding locations of DHPA to the DEP domain were similar among all the simulations, and involved many of the same residues seen in the DEP-POPA simulations. The residues principally responsible for binding DHPA were polar (Lys$^{446}$, Arg$^{472}$, Arg$^{376}$, Lys$^{477}$, Lys$^{484}$, and Asn$^{483}$). This surface is capable of binding to not only the planar surface of the POPA membrane, but also soluble DHPA clustered over the positively charged protein surface.

In each of the three simulations of the Dvl2 DEP domain in the presence of POPA-containing lipid bilayers, initial contacts between the protein and the lipids were made through residues in the loop region comprising residues 491–497, most frequently Lys$^{494}$. The binding event was further stabilized through the involvement of lysine and arginine residues in the basic helix 3 extending from residues 472–479 of the protein. We could not visualize significant NMR perturbations by the addition of DHPA in backbone amide groups of helix 3 (Fig. 2). We suggest that changes may occur on their side chains, which cannot be observed in the frequency range tested in our NMR experiments. We demonstrated that single mutations of basic residues drastically reduced PA binding. This observation is in agreement with PA binding of mTOR and Epa1c, in which their residues Arg$^{2109}$ and Arg$^{87}$, respectively, are strictly required for lipid recognition (40,46,47). Interestingly, the mTOR Arg$^{2109}$ side chain, but not its backbone amide, is perturbed by DHPA (47), a behavior that is likely observed for Lys$^{477}$ in the Dvl2 DEP domain.

We biochemically demonstrated that PA binding of the Dvl2 DEP domain is pH-dependent, consistent with the proposed mechanism of Dvl membrane binding (20), and generally accepted for other PA recognition modules (45). Thus, differentially protonated states of the lipid will determine the interaction with surface-basic residues of the protein through electrostatic interactions. Membrane binding of a protein to PA, through its basic residues, introduces additional positive charges at the membrane. This association will decrease the proton content due to charge repulsion and, thus, promotes an increment of the local pH at the membrane. The second $pK_a$ value for PA is between 6.9 and 7.9 (48), and, consequently at pH values higher than its second $pK_a$ value, the majority of the lipid will be deprotonated and more prone to protein binding. Experimentally, we observed this phenomenon; increasing the pH value in the Dvl2 DEP domain NMR sample led to specific chemical shift changes that were also observed after the addition of DHPA.

In addition to PA, Wnt signaling-related proteins including Prickle, Rac1, and the muscle kinase receptor

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have been shown to interact with the Dvl DEP domain by pull-down or immunoprecipitation assays (6,49,50). However, there is a lack of information about the mechanism of recognition of the complexes, their kinetic properties, and whether PA plays a role in these associations. Thus, the availability of the resonance assignments of the Dvl2 DEP domain and details of PA binding will provide the tools to monitor these associations at atomic resolution, map their binding sites, and understand how Dvl controls the equilibrium and divergence of multiple Wnt-dependent signaling pathways by its DEP domain.

CONCLUSIONS

We report details of the structural and functional basis of PA recognition by the Dvl2 DEP domain. The work shown here reveals distinctive features that have escaped previous attention, including that, in addition to helix 3, a loop between the β3 and β4 elements plays a role in PA recognition. Local conformational changes accompanying binding and likely involve helix 2 and β-strand 4, both of which are tightly associated with the hydrophobic core of the DEP domain, which is important to maintain tertiary structure stability (17). More significantly, we demonstrated that mutations on single conserved basic residues in the DEP domain affect lipid binding, a property that has been found in other PA binding proteins (40,46,47). Given the modest affinity of the DEP domain to PA, additional molecular interactions should be present (e.g., Fz binding, ligation to other acidic phospholipids) for the formation of a stable Dvl membrane-binding complex. Furthermore, our identification of pH-dependent binding of the Dvl2 DEP domain not only supports the electrostatic/hydrogen-bond switch model (38), but also confirms recent findings that indicate that the association should be tightly modulated by changes in the intracellular pH, by the action of proton pumps (20) and, perhaps, by fluctuations in PA levels at the plasma membrane.

SUPPORTING MATERIAL

Four tables, 10 figures and references (51–53) are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(14)00133-7.

We thank Dr. Janet Webster for assistance during preparation of the manuscript and Dr. Michael Overduin for his initial support of this work. Additional thanks go to Advanced Research Computing at Virginia Tech for computing time. We are also grateful to Dr. Hugo Azurmendi, Abigail Ellis, Karla Sanchez, and My-Thanh Vo, for their contribution in the initial phase of this work.

This work was supported by the National Institutes of Health (grant No. R03HD065999 to D.G.S.C.).

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Dvl2 DEP Binding to Phosphatidic Acid


CONTRIBUTION TO THE PROJECT

In this paper, we described a protocol for obtaining isotopically labeled peptides, which I used to purify $^{15}$N- and $^{13}$C-labeled TIRAP PBM. For this project, I tracked the overexpression of Dab2 SBM, and contributed to generate the table, properties of representative peptides purified from bacteria.
A rapid procedure to isolate isotopically labeled peptides for NMR studies: application to the Disabled-2 sulfatide-binding motif

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A procedure for obtaining isotopically labeled peptides, by combining affinity chromatography, urea-equilibrated gel filtration, and hydrophobic chromatography procedures, is presented using the Disabled-2 (Dab2) sulfatide-binding motif (SBM) as a proof of concept. The protocol is designed to isolate unstructured, membrane-binding, recombinant peptides that co-purify with bacterial proteins (e.g., chaperones). Dab2 SBM is overexpressed in bacteria as an isotopically labeled glutathione (GST) fusion protein using minimal media containing [¹⁵N] ammonium chloride as the nitrogen source. The fusion protein is purified using glutathione beads, and Dab2 SBM is released from GST using a specific protease. It is then dried, resuspended in urea to release the bound bacterial protein, and subjected to urea-equilibrated gel filtration. Urea and buffer reagents are removed using an octadecyl column. The peptide is eluted with acetonitrile, dried, and stored at ~80 °C. Purification of Dab2 SBM can be accomplished in 6 days with a yield of ~2 mg/l of culture. The properties of Dab2 SBM can be studied in the presence of detergents using NMR spectroscopy. Although this method also allows for the purification of unlabeled peptides that co-purify with bacterial proteins, the procedure is more relevant to isotopically labeled peptides, thus alleviating the cost of peptide production. Copyright © 2014 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: detergent; disabled-2; isotopes; NMR; recombinant peptide; urea

Introduction

Peptides are involved in a variety of biological processes acting as antimicrobial macromolecules, enzyme inhibitors, and signal transduction triggers. Peptide purification methodologies have improved over the last few years by reducing the amount of time and effort required to obtain intact, biologically active peptides suitable for structural studies. Initially, peptide purification technologies (molecular mass <10,000 Da) involved selective precipitations and adsorption of peptides along with ion-exchange and gel filtration chromatography, which led to low yields; more advanced technology involving the use of reversed-phase HPLC has led to higher yields [1]. Reversed-phased HPLC is carried out using sequential chromatography on columns containing different classes of silica-based packing materials, with octadecyl (C₁₈) columns being the most popular because they are suitable for the purification of hydrophilic peptides. For hydrophobic peptides, however, C₆ and diphenyl columns are recommended [1]. These columns use acetonitrile as a solvent because of its low viscosity, low absorbance at 214 nm (which is important for detecting the peptide bonds), and relatively high volatility. However, the HPLC technology has limitations, including low yield in the purification of extremely hydrophilic peptides, which show low retention in most of the HPLC-based columns; acetonitrile-mediated denaturation; and insolubility of polypeptides [2]. Although peptides can easily be produced by chemical synthesis, the large quantities required for structural or functional assays make this method cost-ineffective, resulting in their overexpression in a heterologous system as the default option. Although many peptides can be overexpressed as fusion proteins using Escherichia coli and other expression systems, the major limitation is their aggregation propensity, resulting in a low solubility behavior. Expression, yield, stability, and solubility of peptides were successfully tackled when maltose-binding protein [3], E/K coils [4], and a combination of the B1 immunoglobulin domain of streptococcal protein G (GB1) and His tags were employed [5]. Antimicrobial peptides, which are ~50 amino acids in length, exhibit more unusual properties, such as being hydrophobic with an excess of positive charges, making them difficult to isolate [6]. Thioredoxin, GB1, and glutathione S-transferase (GST) are the most popular carrier proteins for fusion expression with peptides [7,8]. The reason for their popularity resides in their ability to increase the solubility of fusion proteins, with thioredoxin being most favorable because of its small size (11.8 kDa) and because of its role as a chaperone in facilitating protein folding [9]. Nuclear magnetic resonance (NMR) spectroscopy is a powerful technology that provides detailed information about the...
structure, function, and dynamics of macromolecules. To obtain such information, NMR depends on the presence of stable isotopes in peptides, including carbon ($^{13}$C) and nitrogen ($^{15}$N). Chemical synthesis of isotopically stable peptides is expensive; the cost is associated with both the amount and length of the peptide to be studied. Thus, isotopic enrichment of media for bacterial growth and peptide overexpression is used, as such media can easily be manipulated by the addition of specific isotopes. Indeed, $[^{13}$C] glucose and $[^{15}$N] ammonium chloride are commonly used as the sole source of carbon and nitrogen, respectively, and usually help to generate milligram amounts of isotopically labeled proteins [10].

Here, we report on an optimized protocol to isolate peptides, which, because of their unstructured nature, often co-purify with host bacterial proteins such as chaperones. As an example, we describe the purification of a membrane-binding peptide derived from Dab2, a protein that is best known for modulating the extent of platelet–platelet and heterotypic interactions [11]. Dab2 exerts such functions by interacting with platelet sulfatides [12] via a short helical and amphipathic membrane-binding region that we have recently characterized as necessary and sufficient for negatively regulating platelet aggregation events [13]. This region, which we named Dab2 SBM, has been isolated for structural studies by a recently characterized as necessary and sufficient for negatively regulating platelet aggregation events [13].

Materials and Methods

Materials

The following is a list of chemicals used and their suppliers: restriction endonucleases, T4 DNA ligase, aprotinin, and acetonitrile (Fisher Scientific; Fair Lawn, NJ, USA); leupeptin and pepstatin (G-Biosciences; St. Louis, MO, USA); DL-dithiothreitol (DTT), isopropl-$\beta$-thio-galactopyranoside (IPTG), urea, benzamidine hydrochloride, and Luria Bertani (LB) medium (Research Products International; Mt. Prospect, IL, USA); Triton X-100, TFA, formic acid, BSA, and sodium azide (Fisher Scientific; Fair Lawn, NJ, USA); leupeptin and pepstatin (Fisher Scientific; Fair Lawn, NJ, USA); lysozyme (MP Biomedicals; Solon, OH, USA); and brain sulfatides (Avanti Polar Lipids; Andover, MA, USA). A peptide representing Dab2 residues 24–58 was chemically synthesized by Biopeptide Co., Inc. (San Diego, CA, USA).

Cloning, Expression, and Purification of Dab2 SBM

A cDNA comprising the human Dab2 SBM (residues 24–58) was cloned into a pGEX6P1 vector (GE Healthcare; Pittsburgh, PA, USA). The cDNA region was amplified using the primers 5′-CGCGGATCCCTCAAAGAAGGA-AAAAAAGAAAGGCCC 3′ (forward) and 5′-CGCGAATTCATGCAATGCAGCTGCGCC 3′ (reverse) and by ligation of the PCR product to linear pGEX6P1 using sticky ends generated by the restriction enzymes BamHI and EcoRI. On the basis of this cloning strategy, the recombinant peptide is expected to have five extra amino acids from the vector at its N-terminus after protease cleavage. The recombinant plasmid was transformed into Rosetta E. coli cells (Stratagene). E. coli LB pre-cultures were inoculated from a glycerol stock of transformed cells. Preparative LB cultures were obtained by a 1:50 dilution of pre-cultures in shaking flasks and incubated at 37 °C until the media reached an optical density of $\sim$0.8. Induction of the GST fusion peptide resulted from the addition of 1 mM IPTG followed by incubation for 4 h at 25 °C. Induced bacterial cells were harvested by centrifugation at 9,600 g for 10 min at 4 °C and subjected to two freeze-thaw cycles to facilitate the extraction of the fusion peptide during purification. The cell pellet was resuspended in an extraction buffer (50 mM Tris–HCl, 500 mM NaCl, 1 mM DTT, pH 7.3) containing a protease cocktail represented by 1 μM leupeptin, 1 μM pepstatin, 1.7 μg/ml aprotinin, and 1 mg/ml benzamidine and then lysed by the addition of 0.1 mg/ml lysozyme and 0.5% Triton X-100 and incubated for 20 min at 4 °C. The resulting crude extract was subjected to sonication (using a Branson model 250 sonicator; Cleveland, OH, USA) on ice at 30% amplitude with eight 30-s bursts and 30-s cooling intervals between bursts. The lysate was clarified at 9,600 g for 60 min at 4 °C. The supernatant was stirred with glutathione beads (2 ml of wet beads/l of culture) for 60 min at 4 °C. Beads were then washed four times with a washing buffer (50 mM Tris–HCl, 500 mM NaCl, 1 mM DTT, 1 mM NaN$_3$, pH 8). The GST fusion peptide, bound to glutathione beads, was cleaved by incubation with PreScission protease (0.05 units of protease/μg of protein; GE Healthcare) for 3 h at room temperature. Untagged Dab2 SBM, which was bound to a bacterial protein (likely a chaperone) was then concentrated to dryness using a centrifugal evaporator (Savant SpeedVac; Farmingdale, NY, USA). The dried protein film was then suspended in 6× urea for 30 min at room temperature and subjected to a fast protein liquid chromatography-driven size-exclusion chromatography using a Superdex 200 column (GE Healthcare), equilibrated with 50 mM Tris–HCl, 250 mM NaCl, and 3 mM urea, pH 8.0. Fractions containing free Dab2 SBM were pooled and dried again. The peptide film was then resuspended in a minimum volume of 0.1% TFA, acidified to a pH equal to or less than 3.0 by the addition of a small amount of formic or acetic acid, and loaded onto a C$_{18}$ column (Waters Sep-Pak; Milford, MA, USA) previously equilibrated with 2% acetonitrile and 0.1% TFA. The column was washed twice with 2% acetonitrile and 0.1% TFA to remove salt and urea, and the peptide was eluted with 75% acetonitrile. Dab2 SBM was dried again as described earlier and stored at $\sim$80 °C until use. The purity of Dab2 SBM (~1 ng peptide in 75% acetonitrile) was estimated by mass spectrometry analysis using an Applied Biosystems Grand Island, NY, USA; model 4800 MALDI TOF/TOF mass spectrometer at the Virginia Tech Mass Spectrometry Incubator Facility. Peptide concentration was estimated by the bicinchoninic acid method [14]. The molecular mass of the isotopically labeled peptide was estimated using the Exasy Bioinformatics Resource Portal (web.expasy.org/compute_pl/). For the preparation of labeled peptides, the procedure was essentially the same except that bacterial LB cultures were first obtained in shaking flasks by a 1:10 dilution of LB pre-cultures to a volume of 100 ml of minimal media incubated for 1 h at 37 °C followed by another 1:10 dilution to the rest of the minimal media containing [15]$^N$ ammonium chloride as the source of nitrogen and incubated at the conditions described above. Protein electrophoresis analyses were performed using 15% acrylamide Laemmli gels. Bands were identified using Coomassie blue staining and prestained molecular weight markers (Bio-Rad) as protein standards.
NMR Spectroscopy

Nuclear magnetic resonance measurements of either unlabeled or uniformly $^{15}$N-labeled Dab2 SBM in 90% H$_2$O/10%$^2$H$_2$O and 10 mM d$_4$-citrate (pH 5), 40 mM KCl, and 1 mM NaN$_3$ with either 40 or 200 mM d$_{38}$-DPC (NMR buffer) were obtained using 600 MHz Avance NMR spectrometer (Bruker Biospin; Billerica, MA, USA, at the Virginia Tech NMR Facility) equipped with a pulse field gradient triple resonance TBI probe at 25 °C. $^{15}$N-edited HSQC spectrum analyses were carried out by preparing 200 μM of the $^{15}$N-labeled peptide in an NMR buffer, whereas NOESY spectra were obtained using unlabeled Dab2 SBM with a mixing time of 200 ms. NMR

Figure 1. (a) SDS-PAGE analysis of recombinant GST–Dab2 SBM expressed in E. coli at the indicated time points after IPTG induction. (b) Analysis of the purification of GST-Dab2 SBM at different stages using SDS-PAGE. Lane 1, crude extract; lane 2, supernatant after centrifugation of the crude extract; lane 3, bacterial proteins that did not bind to glutathione beads; lane 4, purified GST–Dab2 SBM that co-purified with a high-molecular-weight bacterial protein.

Figure 2. (a) Gel filtration analysis of Dab2 SBM. A Dab2 SBM/bacterial endogenous protein was loaded onto a fast protein liquid chromatography-driven Superdex 30 column under native (dotted line) and denaturing (solid line) conditions using a buffer containing 3 M urea as described in the Materials and Methods section. Inset, top: amino acid sequence corresponding to the recombinant Dab2 SBM. Bolded residues represent the two sulfatide-binding motifs in Dab2. Amino acids from the vector are GPLGS. Inset, bottom: SDS-PAGE analysis of the peptide purification. Lane 1, molecular mass markers; lane 2, synthetic Dab2 SBM; lane 3, endogenous bacterial protein from a fraction of the first peak of elution; lanes 4–9, fractions corresponding to the second peak of the chromatogram containing the purified Dab2 SBM peptide. (b) MALDI TOF analysis of purified untagged uniformly $^{15}$N-labeled Dab2 SBM.
Results and Discussion

Dab2 SBM Expression and Purification

The structural and sulfatide-binding characterization of Dab2 focused on SBM because this region binds sulfatides and associates with the surface of platelets to modulate their aggregation [13]. The Dab2 SBM peptide was expressed as a GST fusion protein in E. coli cells after IPTG induction. The SDS-PAGE analysis indicated that the protein stably overexpressed over a course of 4 h at 25 °C (Figure 1a). The GST–Dab2 SBM fusion protein can easily be purified by conventional affinity chromatography using glutathione beads. Purification requires the presence of a cocktail of protease inhibitors given the unstructured nature of the peptide. After glutathione chromatography purification, we observed two bands, one at about 29 kDa, which corresponds to GST–Dab2 SBM, and another at about 80 kDa, which represents a bacterial protein, presumably a chaperone bound to the unfolded peptide (Figure 1b). Because of their high level of expression and the unstructured nature of the peptides, they usually co-purify with bacterial chaperones. Several protocols have been published to avoid purification of recombinant bacterial proteins with endogenous bacterial chaperones. For example, removal of the contaminating chaperone GroEL from GST fusion proteins can be achieved by the addition of denatured proteins to the bacterial lysate prior to the addition of glutathione beads [17]. Similarly, removal of undesired E. coli Hsp70 molecular chaperones (e.g., DnaK) can be obtained by a combination of two approaches: (i) choosing the amino acids surrounding and belonging to the cloning site on the basis of an algorithm to decrease the interaction of the fusion protein with the chaperone and (ii) washing the fusion protein bound to the beads with ATP, MgCl₂, and soluble denatured E. coli proteins before the elution of the protein of interest [18]. In our experiments, size-exclusion chromatography analysis indicated that, after GST removal, the peptide co-eluted in a peak with a high-molecular-weight bacterial protein, with both proteins being found in the void volume of the column (Figure 2, dotted line, and data not shown). However, resuspension of the peptide sample in 6 M urea and further fractionation of the sample using a size-exclusion column, previously equilibrated with a buffer containing 3 M urea, allowed for the release of Dab2 SBM from the bacterial protein (Figure 2a, solid line) with purity at the same level of a peptide that was generated synthetically (Figure 2a, inset).

The purified peptide can be easily subjected to solvent exchange and removal of both urea and salt using a C₁₈ column. Dab2 SBM resulted in a very stable peptide with no obvious indication of degradation based on mass spectrometry analysis of the peptide in 75% acetonitrile (Figure 2b). Indeed, the estimated molecular mass of the ¹⁵N-labeled peptide from mass spectrometry analysis was 4,446.43, close to that estimated from its amino acid sequence (4,445.46). Of note, there have been some reported cases in which the isolated peptides are accompanied by some impurities. For example, the purification of the endothelin-3 peptide using a C₁₈ column cannot be achieved in a single loading of the peptide into the column; instead, a C₁₈ chromatography of the leading edge fractions of the first C₁₈ peak is recommended [19]. A work flow diagram of the purification protocol is indicated in Figure 3. Thus, to our knowledge, the methodology shown in this manuscript represents a novel approach for purifying soluble and intact peptides from host endogenous bacterial proteins using a combined urea treatment and sequential size-exclusion and hydrophobic chromatography procedures.

Expression of untagged peptides in bacteria usually does not work well because of their proteolysis, low yield, and/or toxicity. Fusion peptides not only offer protection of recombinant peptides from endogenous proteases during their overexpression in bacteria but also can be fused by a single or combination of a variety of tags and the introduction of proteolytic and/or chemical cleavage sites to further remove the tag from the recombinant peptide. Accordingly, and to alleviate the cost of production of labeled peptides, additional purification strategies of peptides for NMR studies have been reported using His [20–22], maltose-binding protein [23], the RNA-binding domain of human hnRNP1 [24], GB1 domain [7], ketosteroid isomerase [25], thioredoxin [26,27], and even a combination of tags [20,21,23] yielding high-resolution spectra. Recently, a protocol for the purification of the cysteine-rich heat-stable enterotoxin was developed, in which its three disulfide bonds and proper folding were preserved during purification by the use of human uroguanylin as a tag (Table 1) as demonstrated by the high-resolution HSQC spectrum of the peptide [28]. Protease cleavage often triggers peptide proteolysis and chemical cleavage, such as with cyanogen bromide, and is an alternative option to isolating untagged peptides [29]. However, cyanogen bromide-mediated cleavage is limited to the presence of methionine.

Figure 3. Work flow diagram of key steps described in this report.
residues at the C-terminus of the tag and the absence of internal methionine residues in the target peptide. There is a good rate of success for the purification of fusion peptides from bacterial inclusion bodies (as reviewed in [29]). Despite their high yield, isolation of fusion peptides from these compartments requires additional work and the use of additional chemical denaturants for peptide isolation. The peptide yield obtained using our protocol (~2 mg/l of minimal media) is comparable with those reported for hydrophilic and membrane-binding peptides (Table 1) making a convenient strategy for the isolation of poorly structured peptides at yields required for NMR studies.

NMR Structural and Functional Analysis

Nuclear magnetic resonance spectroscopy is one of the most sophisticated biophysical techniques that provide details of disordered states of proteins and peptides [30]. For membrane-binding peptides, perdeuterated detergents (such as DPC) can promote peptide folding to improve the dispersion of the peptide NMR resonances [31]. DPC is the default detergent of choice because it contains the most common phospholipid head group found in biological membranes that form small micelles suitable for NMR studies. Detergent concentration should be

![Figure 4. Comparison of the HN (a) and Hα (b) chemical shifts of the recombinant Dab2 SBM (opened circles) with the synthetic version of the peptide (filled squares).](image-url)
above its critical micelle concentration [31]. The efficacy of the methodology presented in this report was also demonstrated by comparison of the NH chemical shifts of the recombinant Dab2 SBM with the synthetic version of the peptide in DPC micelles. Under these conditions, both the HN and HA chemical shifts of the recombinant Dab2 SBM are very close to those corresponding to the synthetic peptide (Figure 4). Comparison of the 1H, 15N HSQC spectra of Dab2 SBM in the absence and presence of DPC micelles indicates that Dab2 SBM alone exhibited a random coil conformation in aqueous solution as evidenced by the limited chemical shift dispersion of the backbone resonances of the peptide (7.8–8.6 ppm in 1H), which improved in the presence of DPC (7.65–8.7 ppm in 1H) (Figure 5a), results that are consistent with circular dichroism studies of the Dab2 SBM in the presence of the detergent [13]. Moreover, the superposition of the NH region of the NOESY spectra also showed an improvement in the number of well-resolved resonances when Dab2 SBM was in the presence of DPC micelles (Figure 5b). The presence of NOEs in the NH region is indicative of a helical structure in the peptide. Therefore, the degree of spectral dispersion in the amide region and the presence of additional well-resolved NOESY cross-peaks are evidence of a folded peptide structure. The functionality of the recombinant 15N-Dab2 SBM can be demonstrated by the observation of changes in the NH region of one-dimensional 1H NMR spectra of the DPC-embedded peptide in the presence of a 16-fold excess of sulfatides (Figure 6).

Conclusions

We have demonstrated the feasibility of the isolation of an isotopically labeled recombinant peptide of high purity and good yield. To assess the utility of the protocol presented in this manuscript, N-terminal tagged GST–Dab2 SBM was employed as an example. By using a GST fusion construct, we limited the amount of proteolytic degradation of the peptide in E. coli. Because of its unstructured nature, Dab2 SBM co-purified with an endogenous bacterial protein, presumably a chaperone. The peptide could be purified to homogeneity by incubation of the complex with 6M urea followed by size-exclusion chromatography in 3M urea, followed by hydrophobic chromatography to remove urea. Peptide quantity is sufficient for NMR structural studies in a volume as modest as 1 l of isotopically labeled media. The relevance of Dab2 SBM arises from its ability to negatively modulate platelet aggregation by binding to the platelet surface [13]. Dab2 interacts with platelet surface sulfatides [32], an association that is mediated by the interaction of a short helical basic region that penetrates sulfatide-enriched membranes [33]. The role of Dab2 SBM in membrane binding becomes evident from structural studies that demonstrate that the peptide is unstructured but folds in the presence of DPC micelles. HSQC NMR spectra of Dab2 SBM in the presence of DPC micelles exhibited better dispersion of the peptide resonances (Figure 5) and, more importantly, bound sulfatides (Figure 6). Overall, the protocol presented here provides an alternative strategy for purifying unstructured peptides with a relatively high yield and purity and simultaneously alleviating the costs of generating isotopically labeled synthetic peptides for structural studies.

Figure 5. (a) Two-dimensional 1H–15N HSQC NMR spectra of uniformly 15N-labeled Dab2 SBM (200 μM) in the absence (red) and presence of isotopically labeled DPC micelles (black). (b) Superposition of the HN–HN region of 2D NMR 1H–1H NOESY spectra of Dab2 SBM (1 ms) without (red) and with (black) DPC micelles. Mixing time t_m = 200 ms.

Figure 6. A zoom of the HN region of the one-dimensional 1H NMR spectrum of unlabeled Dab2 SBM showing 1H chemical shift perturbations before (black) and after (red) the addition of 16-fold sulfatides in DPC micelles. Inset: one-dimensional 1H NMR spectrum of sulfatides in 50 mM DPC indicating the absence of any resonances in the HN region.
Acknowledgements

We thank Dr Janet Webster for critical reading and comments on the manuscript and Dr Narasimhamurthy Shanaiha for NMR technical support. This work was supported by the National Science Foundation CAREER (MCB-0844491) and the American Heart Association (11BGIA616000) grants to CVF and DGSC, respectively.

References

Part IV: BACKBONE $^1$H, $^{13}$C, AND $^{15}$N RESONANCE ASSIGNMENTS OF THE TOM1 VHS DOMAIN

CONTRIBUTION TO THE PROJECT

This manuscript has been submitted to the Journal of Biomolecular NMR Assignments. In this manuscript, we report the $^1$H, $^{13}$C, AND $^{15}$N backbone resonance assignments of the VHS domain of human Tom1, which is an adaptor in endosomal trafficking. Tom1 VHS domain associates with ubiquitinated cargo and phosphoinositide in endosomal membrane. I expressed, and purified $^{13}$C, $^{15}$N Tom1 VHS. I helped with the analysis of NMR spectra and with the deposit of the NMR backbone assignments of the Tom1 VHS domain at the Biological Magnetic Resonance Data Bank.
Backbone $^1$H, $^{13}$C, and $^{15}$N resonance assignments of the Tom1 VHS domain

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Abstract

Efficient trafficking of ubiquitinated receptors (cargo) to endosomes requires the recruitment of adaptor proteins that exhibit ubiquitin-binding domains for recognition and transport. Tom1 is an adaptor protein that not only associates with ubiquitinated cargo but also represents a phosphoinositide effector during specific bacterial infections. This phosphoinositide-binding property is associated with its N-terminal VHS domain. Despite its biological relevance, there are no resonance assignments of Tom1 VHS available that can fully characterize its molecular interactions. Here, we report the nearly complete $^1$H, $^{13}$C, and $^{15}$N backbone resonance assignments of the VHS domain of human Tom1.

**Keywords** Tom1, VHS domain, endosome, protein trafficking, ubiquitin, phosphoinositide
**Biological context**

Proper cellular function requires accurate delivery of ubiquitinated cell-surface receptors (cargo) via endocytosis, followed by delivery of this cargo into early endosomes where sorting takes place (Cullen, 2008). Cargo is further sorted and allocated to intraluminal vesicles of late endosomes or multivesicular bodies for later degradation in the lysosomal lumen. Ubiquitination signals the cytosolic proteasome machinery to initiate protein degradation, but it can also lead to initiation of other degradative (such as the lysosomal pathway) and nondegradative pathways (reviewed in (Husnjak and Dikic, 2012)). Ubiquitin can be covalently attached as a single unit (monoubiquitination), conjugated to multiple sites in a tagged protein (multi-monoubiquitination), or attached as a polyubiquitin chain. In the endosomal compartments, cargo delivery is mediated by adaptor protein complexes. The endosomal sorting complex required for transport (ESCRT) machinery is the best-studied molecular complex involved in the sorting of ubiquitinated cargo (Campsteijn et al., 2016). It is also involved in the formation and release of intraluminal vesicles into the endosomal lumen for cargo degradation in lysosomal compartments. The ESCRT machinery is composed of five unique protein complexes named ESCRT-0, -I, -II, -III, and -IV, which associate with each other for cargo sequestering and sorting. In mammals, the ESCRT-0 multimeric complex is composed of adaptor proteins with multiple ubiquitin-binding domains, which are required for early association to ubiquitinated cargo. In addition, several other proteins are structurally related to ESCRT-0 and have been suggested to participate in early transport of ubiquitinated cargo receptors. The endosomal adaptor proteins Toll-
interacting protein (Tollip) and target of Myb1 (Tom1) are considered alternative ESCRT-0 proteins because they specifically sort the innate immune interleukin-1 receptor (Brissoni et al., 2006) and the transforming growth factor-type I receptor (Zhu et al., 2012) to the lysosomal degradation pathway. Tollip, via its C2 domain, associates with endosomal membrane domains enriched with phosphatidylinositol 3-phosphate (PtdIns(3)P) (Ankem et al., 2011) and both Tollip C2 and CUE domains bind ubiquitin (Mitra et al., 2013). Tom1 is recruited to endosomal compartments by interaction with Tollip (Katoh et al., 2004; Yamakami et al., 2003) and this association inhibits binding of Tollip to PtdIns(3)P (Xiao et al., 2015), suggesting that this may be necessary for Tollip to commit to cargo recognition. Tom1 presents two ubiquitin-binding domains known as the N-terminal Vps27, Hrs, STAM (VHS), and the central GGA and Tom1 (GAT) domains. The Tom1 GAT domain is a helical module (Xiao et al., 2016) that mediates Tom1-Tollip interaction and binds ubiquitin, whereas the VHS domain associates with cargo by binding to ubiquitin (Ren and Hurley, 2010). The Tom1 VHS domain has recently been shown to bind endosomal signaling PtdIns(5)P, delaying epidermal growth factor receptor degradation and fluid-phase bulk endocytosis in a bacterial infection model (Boal et al., 2015). The crystal structure of the Tom1 VHS domain displays a basic patch located on helix 3 (Misra et al., 2000), which seems to be important for PtdIns(5)P binding as determined by a site-directed mutagenesis approach (Boal et al., 2015). Here, we report the nearly complete resonance assignments of the human Tom1 VHS domain, providing a means to study its molecular interactions at high resolution.
Methods and experiments

Protein expression and purification

The cDNA encoding Tom1 VHS (representing residues 11-149) was obtained from a human liver cDNA library by PCR and primers (forward, 5’ CGCGGATCCTCTCCAGTGGGACAGCGCATC GAGAAAGCC 3’; reverse, 5’ GGAATTCCTAGCCTTTCCTCCGCAGGTCTC 3’) that contained the BamH1 and EcoR1 restriction sites for subcloning into the pGEX4T3 vector. The recombinant $^{12}$C, $^{15}$N-labeled GST-Tom1 VHS fusion was expressed in *E.coli* (Rosetta strain), which was grown in minimal media supplemented with $^{15}$NH$_4$Cl and $^{13}$C-U-glucose, (Cambridge Isotope Laboratory Inc.) as the source of nitrogen and carbon, respectively. Induction of the GST-tagged fusion Tom1 VHS was performed by the addition of 1 mM IPTG to the bacterial cell culture at an OD$_{600}$ of ~0.8 followed by a 4 h incubation at 25°C. Cell pellets were suspended in ice-cold equilibrium buffer containing 50 mM Tris-HCl (pH 7.3), 500 mM NaCl, 0.1 mg/ml lysozyme, 1 mM DTT, 5 mM benzamidine, and 0.1 % Triton X-100. The suspension was further processed by sonication and centrifugation and the resulting supernatant was incubated with glutathione affinity beads (GE Healthcare). Bound GST-Tom1 VHS was cleaved by incubation with thrombin in a buffer containing 50 mM Tris-HCl (pH 7.3), and 500 mM NaCl overnight at room temperature. Protein was recovered in the same buffer with the addition of 5 mM DTT, and concentrated using a 3-kDa-cut-off concentrator device (Millipore) and further purified by an ÄKTA FPLC system using a Superdex 75 column (GE Healthcare), previously equilibrated with 20 mM Tris-HCl (pH 8), 500 mM NaCl, and 1 mM DTT.
Protein fractions were pooled, concentrated and exchanged with a buffer containing 20 mM $d_{11}$-Tris-HCl (pH 7), 50 mM NaCl, 5 mM $d_{18}$-DTT, and 10% D2O. Protein concentration was calculated using the bicinchoninic acid method.

**NMR experiments**

NMR experiments, which assigned backbone $^1$H, $^{13}$C, and $^{15}$N resonances of the Tom1 VHS domain (0.8 mM) were recorded at 25°C on a Bruker Avance III 600 MHz NMR spectrometer (Virginia Tech) equipped with a 5 mm z-gradient triple resonance probe using TopSpin 3.5 software and a nonuniform sampling method. Sequential assignments of the backbone $^1$H, $^{13}$C, and $^{15}$N resonances were made from $^1$H, $^{15}$N-HSQC, CBCA(CO)NH, HNCACB, HNCA, and HNCO experiments (Grzesiek et al., 1993; Muhandiram and Kay, 1994). Data was processed using NMRPipe (Delaglio et al., 1995) and analyzed using Sparky (Goddard and Kneller, 2008), NMRview (Johnson and Blevins, 1994), and the CCPNMR (Vranken et al., 2005) software.

**Assignments and data deposition**

The $^1$H, $^{15}$N-HSQC spectrum of Tom1 VHS (Fig. 1a) exhibits widely dispersed resonances with 93% of backbone $^{15}$N and $^1$H$^N$ resonances assigned along with 94%, 95%, and 93% of $^{13}$C, $^{13}$C, and $^{13}$CO resonances, respectively. Resonance assignments of residues Gly14, Gln15, Gln26, Thr43, His83, Leu104, Pro109, Pro110, Arg129, and Leu149 are incomplete. Assignments for Ser11, Glu44, Glu45, Gly46, Ser130, and Ser131 were not obtained. These unassigned residues are located in loop regions as observed from the crystal structure of the protein (Misra et al., 2000). The Tom1 VHS secondary structure prediction scores (Fig. 1b), using TALOS+ (Shen et al., 2009), are
in good agreement with those secondary structural elements reported for the crystal structure of the protein (Misra et al., 2000). The chemical shift values from the $^1$H, $^{13}$C, and $^{15}$N resonances of Tom1 VHS have been deposited in the BioMagResBank under accession number 26806.

Acknowledgements

We thank Dr. Janet Webster for assistance during preparation of the manuscript. This work was funded by an American Heart Association Grant-in-Aid (13GRNT16960080) to D.G.S.C.

Conflict of interest

The authors declare that they have no conflict of interest.
References


Fig. 1  

a $^{1}\text{H},^{15}\text{N}$-HSQC spectrum of the human Tom1 VHS domain at pH 7 and 25°C. Backbone $^{1}\text{H},^{15}\text{N}$ chemical shifts are labeled with their residue assignments. Inset: An overlapped region of the HSQC spectrum of the protein is magnified.  

b Cartoon representation of the secondary structural elements obtained from the crystal structure of Tom1 VHS (top) and those scored using TALOS+ (bottom).


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