Sulfatides mediate Disabled-2 membrane localization and stability during platelet aggregation

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Thrombosis, the major cause of heart attack and strokes, is triggered by localized clotting of the blood as the result of deregulated platelet aggregation. During the repair of vascular injury, clotting usually occurs when platelets adhere to each other at the site of vascular injury in order to stop bleeding. Distinct protein receptors and adhesive ligands together with the blood flow conditions govern this process. One of the negative regulators in platelet aggregation is Disabled-2 (Dab2), a modular protein that is released upon platelet activation to the extracellular platelet surface. Dab2 inhibits platelet aggregation through its phosphotyrosine-binding (PTB) domain by competing with fibrinogen for $\alpha$II$\beta$3 integrin binding on the activated platelet surface. Sulfatides are also found on the platelet surface, interacting with adhesive and coagulation proteins and, thus, they are thought to play a major role in haemostasis and thrombogenesis.

Here, we show that the Dab2 PTB domain specifically interacts with sulfatides through two conserved basic motifs. The sulfatide-binding site overlaps with that of phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P$_2$) in the PTB domain. Whereas sulfatides recruit the Dab2 PTB domain to the platelet surface, thus sequestering the protein from thrombin-mediated platelet aggregation, the phosphoinositide mediates its internalization. Experimental data support the hypothesis that two pools of Dab2 co-exist at the platelet surface and that the balance between them controls the extent of the clotting response.
Dedication

As my gift back to Grandpa, whose silent prayers, generous love, and courageous example have blessed me in my work, play, and study more than he will ever know.

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Attributions

Those who have directed and contributed to the work presented in various chapters of this dissertation are listed here, with a brief description of their involvement.

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Publication in preparation

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Abbreviations

A alanine
ADP adenosine di-phosphate
AP-2 adaptor protein-2
APP amyloid precursor protein
ATP adenosine tri-phosphate
BSA bovine serum albumin
CD circular dichroism
CME clathrin-mediated endocytosis
DAG diacylglycerol
Dab disabled
DH Dab homology
DMSO dimethyl sulfoxide
Dvl dishevelled
GP glycoprotein
Grb2 growth factor receptor bound protein-2
GST glutathione S-transferase
IP_{3} inositol triphosphate
K lysine
LDL low density lipoprotein
NPXY Asp-Pro-X-Tyr
PBS phosphate buffered saline
PC phosphatidylcholine
PE phosphatidylethanolamine
PS phosphatidyserine
PF4 platelet factor IV
PH pleckstrin homology
PKC protein kinase C
PLC phospholipase
PRD proline-rich domain
PTB phosphotyrosine binding/interacting domain
PTB^{4M} phosphotyrosine binding/interacting domain K25A/K49A/K51A/K53A
PtdIns(4,5)P_{2} phosphatidylinositaol (4,5)-bisphosphate
PtdIns(3)P phosphatidylinositol 3-phosphate
RGD Arg-Gly-Asp
RGDF Arg-Gly-Asp-Phe
RGDS Arg-Gly-Asp-Ser
RGE Arg-Gly-Glu
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPR surface plasmon resonance
TGFβ transforming growth factor β
TRAP thrombin receptor-activating peptide
VWF von Willebrand factor
Wnt wingless type
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Chapter 1: Background and Significance

A. Disabled-2 (Dab2)

Disabled-2 (Dab2) acts as an adaptor protein in multiple pathways, including endocytosis \(^8\), \(^9\) and canonical Wingless Type (Wnt) signaling. \(^10\), \(^11\) Dab2 also negatively regulates integrin-dependent platelet aggregation. \(^12\) Dab2 contains a N-terminal Phosphotyrosine Binding/Interacting Domain (PTB) which binds phospholipid and peptide ligands. \(^13\) Thus, abrogation of Dab2 peptide and lipid binding function can be predicted to disrupt the delicate checks-and-balances system governing vesicular lesion responses and arterial thrombus formation.

A.1 Domain architecture of Dab2

Structurally, Dab2 contains two functionally relevant domains: the N-terminal PTB domain and a C-terminal proline-rich domain (PRD) (Figure 1.1). \(^13\), \(^14\) The PRD domain inhibits mitogenic Ras monomeric GTPase pathway activation by binding to the Growth factor Receptor Bound protein-2 (Grb2). \(^15\) The Dab2 PTB domain is a member of the Dab Homology (DH) domain family, which mediates binding to specific peptides and lipids. \(^13\) The PTB domain mediates Dab2 interaction with Smad2 and Smad3 in the Transforming Growth Factor β (TGFβ) pathway as well as Axin and Dishevelled-3 binding in the canonical Wnt pathway. \(^15\) The Dab2 PTB domain specifically binds phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P\(_2\)) as well as unphosphorylated Asp-Pro-X-Tyr (NPXY) peptide sequences. \(^13\), \(^16\)

![Figure 1.1: Domains of Dab2. Dab2 consists of an N-terminal PTB domain and a C-terminal PRD. Red bars indicate lysine (K) residues 53 and 90, which mediate PTB-PtdIns(4,5)P\(_2\) binding.\(^{13}\) ]
Interestingly, the PTB domain found in Disabled-1 (Dab1) and Dab2 contains two separate binding pockets for the binding of peptides and phosphoinositides based on structural analysis of ligand binding to the Dab1 PTB domain.\textsuperscript{13} Crystallographic analysis shows that the PTB peptide binding site is located on the side of a $\beta$-barrel on the opposite side of the lipid binding site, approximately 25 Å apart (Figure 1.2A).\textsuperscript{13} For Dab2, lipid binding is mediated by Lys53, Lys90, Arg84, His89, Arg132, and Lys150, which stabilize the phosphate groups of phosphatidylinositol ligands (Figure 1.2B, blue and yellow).\textsuperscript{13} Residues Ser122, His144, and Phe166 are integral for binding of the Amyloid Precursor Protein (APP) NPXY peptide, as demonstrated by mutagenic studies, and residues Glu141, Gly139, Lys163 also stabilize the peptide in the binding pocket (Figure 1.2C, green and pink).\textsuperscript{13} The peptide and lipid binding pockets of the Dab1 PTB have been shown to be energetically independent,\textsuperscript{17} and the Dab1 PTB has been co-crystallized with peptide and lipid ligands.\textsuperscript{13} These data suggest that Dab2 could simultaneously bind both protein partners as part of a signaling cascade and lipid molecules, potentially regulating Dab2 subcellular localization.

### A.2 Dab2 lipid ligands

Dab2 has been shown to bind to protein and lipid partners, including clathrin, Dishevelled-3 (Dvl-3), Adaptor Protein 2 (AP-2), and PtdIns(4,5)P$_2$ via its PTB domain. Additionally, our biochemical data indicate that Dab2 specifically binds sulfatide ligands through its PTB domain and that the sulfatide binding site overlaps with the PtdIns(4,5)P$_2$ binding site.
i. Phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P$_2$)

The PtdIns(4,5)P$_2$ membrane phospholipid is predominantly located on the cytosolic leaflet of the plasma membrane, but PtdIns(4,5)P$_2$ is also found on the cytosolic leaflet of early endosomes and of golgi phospholipid bilayers (Figure 1.3A).$^{18, 19}$

![Figure 1.3: Chemical structures of (A) PtdIns(4,5)P$_2$ and (B) sulfatide ligands of the Dab2 PTB domain.](image)

The PtdIns(4,5)P$_2$ membrane phospholipid functions in the localization of myosin VI, Phopholipase C- $\delta_1$ (PLC$\delta_1$), Dab1, and Dab2. For example, Dab1 plays an integral role in Reelin signaling during the positioning of neuronal cells in brain development, and PtdIns(4,5)P$_2$ binding is necessary for Dab1 function.$^{20}$ The Dab1 protein also binds to PtdIns(4,5)P$_2$ via an amino-terminal PTB domain, and this interaction mediates Dab1 membrane localization and subsequent phosphorylation.$^{21}$ As another example, the PLC$\delta_1$ enzyme cleaves PtdIns(4,5)P$_2$ into secondary messengers inositol triphosphate (IP$_3$) and diacylglycerol (DAG).$^{18}$ The PLC$\delta_1$ enzyme is strongly recruited to the plasma membrane by PtdIns(4,5)P$_2$ in mammalian cells, which is necessary for its enzymatic function.$^{22}$ Myosin VI also binds specifically to PtdIns(4,5)P$_2$, which localizes the protein to the plasma membrane to participate in endocytic vesicle formation.$^9$ Abrogation of myosin VI/ PtdIns(4,5)P$_2$ binding hinders myosin VI function, shown in immunofluorescence analysis by the reduced the number of endocytic vesicles formed.$^9$ Shc, a proto-oncogenic adaptor protein, also contains a PTB domain that binds PtdIns(4,5)P$_2$ to mediate plasma membrane translocation and facilitate Shc interaction with membrane-bound receptors.$^{13, 23}$ Thus, PtdIns(4,5)P$_2$ plays a role in protein localization of myosin VI, PLC$\delta_1$, and Shc.

The Dab2 PTB domain also specifically binds PtdIns(4,5)P$_2$, which is hypothesized to mediate Dab2 localization to the plasma membrane and subsequent endocytic vesicles.$^9$ Lysine
residues 53 and 90 in the PTB domain mediate Dab2 binding to PtdIns(4,5)P_2 and to the soluble headgroup of PtdIns(4,5)P_2, inositol-1,4,5-P_3. In the PTB domain of Shc, phosphoinositides and phosphopeptides compete for the shared binding site; in Dab2 the separate binding sites potentially allow simultaneous phospholipid and peptide binding.\textsuperscript{13, 22} In conclusion, PTB-mediated binding of PtdIns(4,5)P_2 is a regulatory mechanism employed by Dab2, PLC\(\delta_1\) and Shc proteins.

**ii. Sulfatides**

Sulfatide is a sulfated galactosylceramide synthesized by cerebroside sulfotransferase containing a ceramide backbone with a sulfate head group and two fatty acid tails that vary in length from 18-24 carbons in length.\textsuperscript{24} Sulfatide lipids are located predominantly on the outer leaflet of the plasma membrane in glandular epithelial cells, neuronal cells, erythrocytes, platelets, and pancreatic islet cells. Sulfatides have been shown to regulate protein localization as well as cellular adhesion during platelet activation.\textsuperscript{24, 25} For instance, sulfatides mediate clustering of voltage-gated ion channels in mouse neuronal cells.\textsuperscript{26} Knockout mice incapable of synthesizing sulfatide lipids exhibit abrogated K\(^+\) ion channel localization along axons.\textsuperscript{26} In the absence of sulfatides, the localization of contactin associated protein, an axonal adhesion mediator, is disrupted in neuronal axons. This results in diffuse distribution of the protein and disrupted K\(^+\) ion channel clustering.\textsuperscript{26} Thus, sulfatides can function in the localization of cellular proteins. During platelet aggregation, sulfatides bind to P-selectin, an adhesion protein present on activated platelet membranes, to stabilize the bridge between adjacent platelets.\textsuperscript{27} This suggests that sulfatides can also mediate intercellular adhesion.

**B. Dab2 pathway involvement**

Dab2 functions in multiple cellular signaling pathways as an adaptor protein. Dab2 acts as a scaffolding protein to negatively regulate canonical Wnt signaling, ultimately decreasing \(\beta\)-catenin mediated gene transcription.\textsuperscript{11, 15} Dab2 is required for TGF-\(\beta\) signaling, facilitating phosphorylation of Smad proteins by TGF-\(\beta\) receptors to allow their subsequent nuclear translocation and transcriptional activity.\textsuperscript{28} In the interest of this work, Dab2 inhibits platelet aggregation by antagonizing the interaction of the \(\alpha_{IIb}\beta_3\) integrin receptor with its fibrinogen ligand.\textsuperscript{3} Furthermore, Dab2 functions as an adaptor protein in endocytosis, linking clathrin to the
plasma membrane and linking cargo proteins to myosin VI for internalization.9,29

**B1. Platelet aggregation**

Platelets, an anuclear blood cell derived from a megakaryocytic progenitor, are specialized agents of vascular damage control. Upon damage to the endothelial wall of a blood vessel, the extracellular matrix protein collagen is exposed to the soluble von Willebrand factor (VWF) protein as well as to circulating platelets.30 Collagen initiates platelet activation directly by binding to the platelet α2β1 integrin receptor, allowing subsequent binding to glycoprotein VI.31 Exposed collagen also tethers VWF, promoting subsequent binding of platelets to VWF via their glycoprotein Ib-IX-V receptors, recruiting platelets to the ruptured vessel wall and initiating platelet activation.30 Upon activation, abundant αIIbβ3 integrin receptors bind to the soluble plasma protein fibrinogen, crosslinking platelets together.

Intracellularly, PLCγ2 then cleaves PtdIns(4,5)P2 to generate the secondary messengers inositol 1,4,5-triphosphate and 1,2-diacylglycerol.32 These mediators initiate a calcium efflux from the dense tubular system and activate Protein Kinase C (PKC), respectively.31 Ultimately, the platelets secrete the contents of both α-granules (primary granules) and δ-granules (dense granules) into the extracellular milieu.3 The α-granules are released first from activated platelets. Numbering approximately 80 per cell, α-granules contain proteins such as Platelet Factor IV (PF4), Dab2, VWF, and other receptors and proteins that govern aggregation.33 The δ-granules number three to eight per cell and contain ADP (Adenosine Di-Phosphate), ATP (Adenosine Tri-Phosphate), calcium, and serotonin; release of dense granules occurs slightly later in activation and promotes secondary activation of the platelet.33

Further signaling converges through the cytoplasmic protein talin to induce a conformational change in the αIIbβ3 integrin receptor (inside-out signaling) so that it can bind the fibrous protein fibrinogen with high affinity, linking platelets together in the formation of a fibrous clot.34, 35 Soluble fibrinogen is cleaved by thrombin to form fibrin, which further stabilizes the clot.1 Platelet membrane rearrangements follow, exposing negatively charged phospholipids from the inner leaflet of the plasma membrane on the outer leaflet of the plasma membrane through a “flip-flop” mechanism.36 The newly formed clot prevents blood loss from the damaged vessel wall.
i. Platelet aggregation mediated by $\alpha_{Ib}\beta_3$ integrin

The predominant integrin receptor on the platelet surface is the $\alpha_{Ib}\beta_3$ integrin, and one resting platelet contains approximately 80,000 of these receptors. In the resting state, the $\alpha_{Ib}\beta_3$ integrin is present as a closed conformation, concealing the binding sites for its Arg-Gly-Asp (RGD) containing ligands. Platelet activation via mechanisms including glycoprotein VI, ADP, or the protease thrombin that activate PLC$\gamma$ result in inside-out signaling that changes the orientation of the $\alpha$II beta-propeller and $\beta_3$ subunits to expose their fibrinogen binding sites. Fibrinogen, a fibrous, soluble plasma protein, is the major ligand for the activated, high affinity $\alpha_{Ib}\beta_3$ integrin receptor. The $\alpha_{Ib}\beta_3$ integrin specifically recognizes two RGD motifs in the $\alpha$-chain of fibrinogen: Arg-Gly-Asp-Ser (RGDS) at residues 572-575 and Arg-Gly-Asp-Phe (RGDF) at residues 95-98, allowing crosslinking of activated platelets in the formation of a nascent thrombus. Upon fibrinogen binding, the $\alpha_{Ib}\beta_3$ integrin receptor initiates intracellular signaling (outside-in signaling) through Src and Syk tyrosine kinases to regulate PLC$\gamma$ function and effect lamellipodia formation. In later phases of activation, the $\alpha_{Ib}\beta_3$ integrin receptor drives changes in lipid raft composition, thus indirectly recruiting actin-interacting proteins to the cytoskeleton, and effects fibrin clot retraction.

ii. Role of PtdIns(4,5)P$_2$ and sulfatides during platelet aggregation

PtdIns(4,5)P$_2$ is primarily found on the inner leaflet of cellular membranes, and plays a prominent role in the recruitment of actin regulatory proteins. Thrombin stimulated activation of platelets has been shown to increase the amount of PtdIns(4,5)P$_2$ associated with cytoskeletal components. Outside-in signaling by the $\alpha_{Ib}\beta_3$ integrin recruits the Rac to intracellular lipid rafts, which drives a PtdIns(4)$P_5$-kinase mediated increase in PtdIns(4,5)P$_2$ in the intracellular lipid rafts. PtdIns(4,5)P$_2$ then attracts proteins such as moesin and Arp3, which assist in cytoskeletal rearrangement (filopodia and lamellipodia formation) and actin nucleation and branching, respectively. Cytoskeletal remodeling proteins then help provide the contractile force to initiate clot retraction. Inhibition of the integrin-mediated PtdIns(4,5)P$_2$ increase inhibits normal clot retraction, suggesting a role for PtdIns(4,5)P$_2$ in the recruitment and tethering of proteins to membrane-cytoskeleton junctions in activated platelets.

Sulfatides, on the other hand, compose approximately 20% of both the extracellular leaflet of the plasma membrane and the granule membranes. Upon activation, there is a significant increase in sulfatides on the plasma membrane, and the sulfatides have been shown to
cluster in patches on the activated platelet surface. In fact, the addition of sulfatide micelles to platelets increases agonist-stimulated aggregation by 23%. Sulfatides have been shown to bind P-selectin, a protein that translocates to the outer membrane from α-granules upon activation and degranulation. The binding of sulfatides to P-selectin stabilizes a nascent thrombus by bridging neighboring aggregated platelets. Therefore, sulfatides play a role as a lipid ligand to enhance platelet aggregation.

iii. Dab2 regulation of platelet aggregation

The Dab2 protein is contained with the α-granules of resting platelets, and upon activation, Dab2 binds to the surface of platelets, as demonstrated by flow cytometry and platelet adhesion assays (Figure 1.4). In aggregometer studies, the addition of Glutathione-S-Transferase-PTB (GST-PTB) decreases platelet aggregation, suggesting that Dab2 negatively regulates platelet aggregation. Flow cytometry analysis demonstrates that the αIIbβ3 integrin mediates the interaction of Dab2 with the activated platelet surface. Functionally blocking the αIIbβ3 integrin receptor with monoclonal antibodies or the RGDS peptide inhibits Dab2 binding to platelets. In addition, sequence analysis reveals an RGD site in the PTB domain of Dab2 (residues 64-66). Pull-down experiments demonstrate that mutation of the site to RGE (Arg-Gly-Glu) also abolishes Dab2 interaction with the αIIbβ3 integrin receptor, suggesting that Dab2 competes with fibrinogen for binding to the αIIbβ3 integrin receptor through its RGD motif (Figure 1.4). Thus, Dab2 secreted from the α-granules negatively regulates platelet aggregation via the αIIbβ3 integrin receptor.
Figure 1.4: Dab2 inhibits platelet aggregation. In the resting platelet, Dab2 is contained within the α-granules. Upon activation, Dab2 is released outside of the platelet and competes with fibrinogen for αIIbβ3 integrin receptor binding, decreasing platelet-platelet adhesion and negatively regulating platelet aggregation.3

B.2 Endocytosis

Endocytosis is the cellular mechanism for intake of soluble particles and macromolecules from the extracellular medium. Endocytic models include clathrin-mediated endocytosis, caveolae-mediated endocytosis, and macropinocytosis. Clathrin-mediated endocytosis (CME) is driven by the assembly of clathrin triskelia around the cytoplasmic domains of ligand-receptor complexes in a developing membrane pit.42 Caveolae-mediated endocytosis is characterized by hydrophobic cholesterol-rich microdomains that form invaginations facilitated by integral membrane caveolin proteins, which can act as scaffolding proteins for signaling complex formation at caveolae.42, 43 Macropinocytosis is the actin-mediated formation of irregular, large endocytic vesicles for the transport of solute macromolecules into the cell.42
i. Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is the major endocytic pathway operating continuously in cells for the internalization of receptors, nutrients, antigens, and growth factors. Commonly initiated by ligand-receptor binding and subsequent receptor clustering, CME is driven by adaptor proteins such as AP-2 that recruit clathrin triskelia around an invagination in the plasma membrane. Proteins such as AP-2 and Dab2 bind to the cytoplasmic internalization sequences of transmembrane receptors, to clathrin, and to phosphoinositide lipids in the plasma membrane (Figure 1.5). The clathrin-coated pits bud from the plasma membrane and are pinched off by dynamin. The vesicles then mature into endosomes and ultimately remove the ligands from the internalized receptors as the pH decreases within the vesicle. In platelets, however, clathrin-coated vesicles are routed into the canalicular membrane system and subsequently packaged into α-granules.

Figure 1.5: Dab2 as an adaptor protein in endocytosis. Dab2 binds to PtdIns(4,5)P_2 and to the cytoplasmic tail of endocytic cargo, such as LDL receptors, via the PTB domain, linking plasma membrane-bound components to the myosin VI actin motor protein. The Dab2 PTB domain also interacts with the clathrin binding protein, AP-2, thus mediating the formation of a clathrin-coated pit. It is suggested that the connection to the actin cytoskeleton provides the force necessary to invaginate the membrane in the budding off of the clathrin-coated endocytic vesicle.
ii. Dab2 involvement in clathrin-mediated endocytosis

The Dab2 protein acts as an adaptor protein in clathrin-mediated endocytosis, linking the plasma membrane bound components to clathrin and to the myosin components (Figure 1.5). Dab2 interacts with AP-2, which recruits clathrin to form the clathrin-coated pit for endocytosis. However, Dab2 can function in the absence of AP-2,8 directly binding to the clathrin heavy chain via a putative type I clathrin-box, LVDLN (amino acids 236-240).29 Dab2 also links the cytoplasmic tail of Low Density Lipoprotein (LDL) receptors to myosin VI, an actin motor protein.8, 9 Myosin VI-actin clusters then provide the force to invaginate the membrane in the formation of the nascent clathrin-coated endocytic vesicle.9 The endocytic pathway exemplifies the ability of Dab2 to simultaneously bind phospholipids and proteins. The Dab2 protein binds to PtdIns(4,5)P2 and to the LDL receptor tail NPXY sequence simultaneously at the plasma membrane,9 and abrogating either interaction ablates the function of Dab2 in endocytosis.8

C. Relevance of Dab2 in disease

Arterial thrombosis is the underlying cause of the majority of heart attacks and 80% of strokes.1 Chronic conditions, such as ischemic heart disease, result from the buildup of atherosclerotic plaques, the resulting narrowing of the arteries, and the increased risk of thrombus formation upon rupture of plaques.30 Upon the rupture of atherosclerotic plaques in arteries, thrombus formation is initiated, primarily mediated by platelet aggregation.1 A plethora of drugs for antiplatelet therapy are available for treatment of bleeding disorders and pathological thrombosis, including cyclooxygenase inhibitors (such as aspirin), ADP-receptor inhibitors (such as clopidogrel), and α<sub>IIb</sub>β<sub>3</sub> integrin inhibitors (such as abciximab).45 The success of treatments that intervene during acute thrombosis depend on the timing of their application.1 However, prolonged prophylactic treatment of patients with cardiovascular disease using exogenous compounds has undesirable side effects such as stomach ulcers, increased bleeding, and activation of platelets via the receptors they target, such as the α<sub>IIb</sub>β<sub>3</sub> integrin receptor.1

The α<sub>IIb</sub>β<sub>3</sub> integrin receptor is a common point in numerous pathways initiating platelet aggregation, and thus is an excellent target for anti-platelet therapy.46 However, exogenous compounds that target the α<sub>IIb</sub>β<sub>3</sub> integrin receptor can mimic ligands, triggering the activation of the platelets by initiating outside-in signaling through the integrin receptor.46 In light of this problem, antibodies specific for the active conformation of the α<sub>IIb</sub>β<sub>3</sub> integrin receptor represent a
prominent frontier for antiplatelet therapy. Dab2 has been shown to bind specifically to the activated conformation of the αIβ3 integrin receptor, representing a potential activation-specific antiplatelet agent.

Elucidating the mechanism of sulfatide-PTB binding will better define how the regulators of αIβ3 integrin are themselves regulated in the seeming chaos of the extracellular milieu upon vascular injury. Specifically, our research will demonstrate how sulfatides and PtdIns(4,5)P2 govern Dab2 inhibition of the αIβ3 integrin receptor during platelet aggregation. By defining the intrinsic mechanisms that regulate the major players of thrombosis, such as αIβ3 integrin receptor, our work will facilitate therapy development to selectively target and moderate the function platelet aggregation using inherent cellular mechanisms.
Chapter 2: Specific Aims

The overall goal of our research was to both characterize the biochemical interaction of Dab2 with a novel lipid ligand, sulfatides, and to determine the physiological relevance of this interaction. Using biochemical techniques, we have elucidated the mechanism of sulfatide interaction with Dab2. By exploring the Dab2-sulfatide interaction in the context of platelet aggregation, we have further defined inherent mechanisms that govern platelet aggregation by a checks-and-balances system, specifically lipid-mediated protein localization and stability.

Based on our preliminary data, we hypothesized that sulfatide binding to Dab2 is mediated by the lipid binding pocket of the N-terminal PTB domain, and that this interaction could play a role in Dab2 localization in activated platelets. What residues mediate the interaction of sulfatides with the PTB domain? Are these residues specific to sulfatide binding or are they utilized for interaction with both sulfatide and PtdIns(4,5)P2 lipid ligands? What is the mode of interaction of these two lipids for PTB binding? Furthermore, what is the physiological relevance of Dab2-sulfatide binding in the context of platelet aggregation? How is the interplay of PtdIns(4,5)P2 and sulfatides for the PTB domain mechanistically relevant to the localization of Dab2 in the activated platelet? To address these questions, we have employed qualitative and quantitative lipid binding assays, competition experiments, immunofluorescence microscopy, and functional platelet assays as presented within the following aims:

**Aim I. To characterize the mechanism of Dab2-sulfatide binding.** We utilized liposome binding assays supplemented by protein:lipid overlay assays to determine the residues that mediate sulfatide binding to the Dab2 PTB domain. These residues were also analyzed in the context of PtdIns(4,5)P2 to determine the spatial relation of the lipid binding sites. Mutant constructs were evaluated for structural integrity using circular dichroism spectroscopy. Furthermore, we quantitatively analyzed the real-time binding kinetics for each lipid to the PTB domain using surface plasmon resonance (SPR).

**Aim II. To define the mode of interaction between sulfatides and PtdIns(4,5)P2 for the Dab2 PTB domain.** We performed competition protein:lipid overlay assays by preincubating soluble lipid ligand with the PTB domain before exposing the complexes to the opposite immobilized lipid. We also analyzed the mode of interaction in real time by SPR, flowing preincubated protein-lipid complexes over the opposite immobilized lipid.
**Aim III.** *To determine how sulfatides and PtdIns(4,5)P2 ligands mediate Dab2 stability, localization, and function to ultimately govern platelet aggregation.* Based on existing literature, Dab2 PTB inhibits platelet aggregation by competing with fibrinogen for binding to the αIIbβ3 integrin receptor.³ We first analyzed thrombin proteolysis of PTB protein bound to each lipid. Then, we performed immunofluorescence microscopy to determine localization of endogenous Dab2 and exogenous PTB proteins during platelet aggregation. We used PTB mutant proteins as well as inhibitors to define the mechanisms underlying localization changes mediated by PTB-lipid binding. Finally, platelet adhesion assays were performed to determine the importance of sulfatide-PTB binding for Dab2-mediated antagonization of the αIIbβ3 integrin receptor during platelet aggregation.

The most abundant platelet surface receptor, the αIIbβ3 integrin receptor, is a major player in early clot formation, mediating crosslinking of platelets via fibrinogen. By defining the importance of Dab2-sulfatide binding during clot formation, our work will contribute to the development of novel therapeutic strategies to moderate platelet function during thrombosis using inherent molecular mechanisms to target specific aspects of platelet aggregation while minimizing harmful side effects resulting from drug therapy.
Chapter 3: Results and discussion

Sulfatides mediate Disabled-2 membrane localization
and stability during platelet activation

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Publication in preparation.

Platelets aggregate to form a plug at the site of vascular damage. Upon activation, platelets release both α- and δ-granules that contribute to platelet adhesion, aggregation and activation. These granules contain a variety of proteins including adhesive and plasma proteins, coagulation factors and protease inhibitors. Platelet adhesion is mediated by the formation of the glycoprotein (GP) Ib-IX-V and von Willebrand factor (VWF) complex, which is followed by the activation of members of the integrin family receptors. Activated platelets accelerate the coagulation process by providing a membrane surface necessary for the production of thrombin, which in turn cleaves and activates fibrin from fibrinogen. In addition, thrombin acts as an agonist for platelet activation, adhesion and aggregation. VWF initiates aggregation under high shear with both fibrinogen and fibrin and ultimately leads to the stabilization of the aggregates. Other molecular complexes, such as P-selectin-sulfatides, and the αIIβ3 integrin and its ligands, including fibrinogen and VWF, also mediate platelet aggregation. Consequently, fibrin facilitates the stabilization of the haemostatic plug and the clotting of blood.

Dab2 is not only implicated in controlling platelet aggregation but also in growth factor signaling, endocytosis, and it negatively regulates Wnt signaling pathway. Importantly, Dab2 is considered a putative tumor suppressor found to be decreased in breast, prostate and ovarian carcinomas. Mammals present two Dab orthologs, Dab1 and Dab2, which present binding domains and phosphorylation sites and lack of catalytic domains. Dab proteins share an N-terminal PTB domain and a proline-rich SH3 domain at their C-terminal region, indicating that they function as adaptor proteins. During endocytosis, the PTB domain mediates both Dab2 binding to lipoprotein receptors and preferentially to the phosphoinositide PtdIns(4,5)P2. A recently proposed model suggests that PtdIns(4,5)P2 is likely to be the physiological ligand that...
mediates Dab1 targeting to membranes.\textsuperscript{54} Furthermore, the PTB domain mediates Dab2 binding to the \(\alpha\)II\(\beta\)3 integrin receptor, thus, exerting its negative role in platelet aggregation.\textsuperscript{3}

- **Conserved basic motifs mediate Dab2 PTB interaction with sulfatides.**

  Analysis of the PTB domain amino acid sequence showed the presence of conserved positively charged residues that resembled two characteristic sulfatide-binding sites represented by the XBBXBX and BXBXBX motifs\textsuperscript{55} (where B and X indicate basic and any residue, respectively) (Figure 3.1A). Several homeostatic proteins including laminin, VWF, P-selectin and thrombospondin have multidomain regions that interact with sulfatides. For example, Laminin-1 binds to sulfatides through a region that comprises two XBBXBX and three BXBXBX sequences.\textsuperscript{56} Likewise, a patch of positively charged residues in VWF is responsible for sulfatides ligation.\textsuperscript{6} To test our hypothesis, we analyzed sulfatide binding by the PTB domain in comparison with other sphingolipids commonly found at the plasma membrane. Among sphingolipids, the Dab2 PTB domain preferentially bound to sulfatides (Figure 3.1B). As the Dab2 PTB domain targets biological membranes, we employed liposomes enriched with sulfatides to further investigate the interaction. The Dab2 PTB domain bound to sulfatide-enriched liposomes (Figure 3.1C) as well as to sulfatides immobilized on membrane strips (Figure 3.S1A). Single amino acid mutations to neutral Ala designed in the putative sulfatide-binding site (Lys25, Lys49, or Lys53) or combination of two mutations (Lys25 and Lys49) reduced but did not eliminate sulfatide binding (Figure 3.S1B). However, a substitution of four positively charged residues (Lys25, Lys49, Lys51 and Lys53; Dab2 PTB\textsuperscript{4M}) in the putative sulfatide-binding sites (Figure 3.1A) with Ala led to complete abolishment of lipid binding (Figure 3.1C). The circular dichroism (CD) spectrum of Dab2 PTB\textsuperscript{4M} (as well as single and double mutants of the same protein) did not exhibit significant changes in the secondary structure content as compared with the wild-type PTB, as shown by the consistency of the percentage of \(\alpha\)-helical content (Helix1 and Helix2) and \(\beta\)-strand content (Strand1 and Strand2) (Table 3.1). These data indicate that mutations do not significantly perturb the global fold of the protein (Figure 3.S2 and Table 3.1). Thus, we conclude that the two positively charged motifs in Dab2 PTB domain play a critical role in sulfatide recognition.
• **Dab2 PTB binds sulfatides and PtdIns(4,5)P$_2$ with comparable affinities.**

The Dab2 PTB domain is known to bind phosphoinositides with preference to PtdIns(4,5)P$_2$, independent of the protein interaction site.$^{57}$ Two basic residues (Lys53 and Lys90) have been reported to play a critical role in PtdIns(4,5)P$_2$ recognition.$^{13}$ Therefore, we examined whether these residues could be also critical for sulfatide binding. Mutation in the PTB domain at both Lys53 and Lys90 to Ala (Dab2 PTB$^{K53K90}$) reduced sulfatide binding in about 3-fold (Figure 3.1C) similarly as observed with a mutation at Lys53 (Figure 3.S1B), but a single mutation at Lys90 (Dab2 PTB$^{K90}$) did not (Figure 3.1C). As expected, the Dab2 PTB domain bound to PtdIns(4,5)P$_2$-containing liposomes, whereas PTB$^{K53K90}$ and PTB$^{4M}$ did not (Figure 3.1D). Consistent with these results, lipid-protein overlay assays showed that the sulfatide-binding mutant, Dab2 PTB$^{4M}$, bound very weakly to bind PtdIns(4,5)P$_2$, whereas PTB$^{K53K90}$ did not bind at all (Figure 3.S3A). Conversely, with the exception of the Lys53 mutant, single or double mutations in the sulfatide-binding sites in the PTB domain did not significantly affect PtdIns(4,5)P$_2$ binding (Figure 3.S3B). Mutations in these residues did not alter the secondary structure of the protein, indicating that they specifically abolished lipid binding (Figure 3.S2 and Table 3.1). Overall, these results indicate that Lys53 plays a critical role in both sulfatide and PtdIns(4,5)P$_2$ ligation.

The kinetics of the interaction between the Dab2 PTB domain and the two lipids were investigated by surface plasmon resonance (SPR). The PTB domain exhibited sulfatide binding with an estimated dissociation constant ($K_D$) of $\sim$1.93 x 10$^{-6}$ M (Figure 3.2A, left panel). This affinity is close to that reported for sulfatide binding by the *Escherichia coli* heat-stable enterotoxin b.$^{58}$ Interestingly, the kinetics displayed a reversible binding mode to either immobilized sulfatide or PtdIns(4,5)P$_2$ liposomes, with Dab2 PTB$^{4M}$ displaying retarded association and dissociation compared to wild type Dab2 PTB (data not shown). The wild type PTB kinetic data for both sulfatide and PtdIns(4,5)P$_2$ fit a two-state binding model with conformational change, but did not fit a 1:1 Langmuir binding model, suggesting a more complex interaction of the PTB domain with its lipid ligands. Likewise, the PTB domain bound PtdIns(4,5)P$_2$ with a calculated $K_D$ of $\sim$1.5 x 10$^{-6}$M (Figure 3.2A, right panel). Dab2 PTB$^{4M}$ exhibited a drastically reduced binding to PtdIns(4,5)P$_2$ liposomes (data not shown), confirming that sulfatide and PtdIns(4,5)P$_2$ binding sites overlap in the PTB domain.
- **PtdIns(4,5)P$_2$ competes with sulfatides for Dab2 PTB binding.**

  We next asked whether sulfatides and PtdIns(4,5)P$_2$ compete with each other for binding to the PTB domain using a protein-lipid overlay competition assay. Pre-incubation of the PTB domain with 10-fold excess of PtdIns(4,5)P$_2$ reduced sulfatide binding in at least 60% (Figure 3.2B), whereas a related phosphoinositide, phosphatidylinositol 3-phosphate (PtdIns(3)P) did not compete with sulfatide for PTB binding (Figure 3.S4). This observation was further confirmed by competing sulfatides and PtdIns(4,5)P$_2$ for PTB domain binding using SPR. Pre-incubation of the protein with PtdIns(4,5)P$_2$ reduced the affinity for sulfatides with increasing molar excess of PtdIns(4,5)P$_2$, showing an IC$_{50}$ of ~ 2 µM for Dab2 PTB (Figure 3.2C). However, the IC$_{50}$ was reduced approximately four-fold for Dab2 PTB$_{4M}$ (Figure 3.2C).

  The tertiary structure of the Dab2 PTB domain exhibits a continuous patch of positively charged residues formed by Lys49, Lys51, Lys53 and Lys90 (Figure 3.3A) (Lys25 is not present in the reported Dab2 PTB domain crystal structure$^{13}$). Two experimental observations can explain the competition of the two lipids for PTB domain binding. First, Lys53 is necessary for both sulfatides and PtdIns(4,5)P$_2$ ligation (Figure 3.3A). Second, despite the fact that Lys90 is far from the sulfatide binding motifs, the orientation of its side chain towards the sulfatide-binding site in the tertiary structure of the protein (Figure 3.3A) may impair simultaneous binding of both lipids to the protein.

- **Sulfatide binding protects Dab2 from thrombin proteolysis**

  During platelet activation, Dab2 is released from α-granules and binds to the extracellular region of αIIβ3 integrin on the platelet surface, where it exerts its anti-platelet aggregation activity.$^{3}$ Dab2 is ineffective in inhibiting thrombin-mediated platelet aggregation due to the presence of two thrombin cleavage sites within its PTB domain.$^{3}$ In agreement with previous observations,$^{3}$ thrombin cleaved the PTB domain leaving a ~25kDa protease-resistant product (Figure 3.3B). To understand the role of sulfatides binding by Dab2 during thrombin-mediated platelet aggregation, the PTB domain was pre-incubated with liposomes containing sulfatides and the protein-lipid complexes treated with thrombin. Interestingly, sulfatide-enriched liposomes protected bound PTB from thrombin cleavage (Figure 3.3C). These results suggest that the thrombin cleavage sites in the PTB domain are less exposed in the protein when bound to sulfatides and thus binding may stabilize Dab2 during thrombin-mediated platelet activation. In contrast, PtdIns(4,5)P$_2$ did not protect the PTB domain from thrombin cleavage (Figure 3.3D),
indicating that the phosphoinositide does not have a direct role on Dab2 stabilization during thrombin-mediated platelet aggregation. Indeed, this result is consistent with the platelet subcellular localization of the lipids. Whereas sulfatides are located at the platelet surface and therefore they can protect Dab2 from thrombin cleavage, PtdIns(4,5)P2 is usually found at the cytosolic side of the membrane.

- Sulfatides compete with the αIIbβ3 integrin for PTB binding on the surface of activated platelets.

The PTB domain has been recently shown to bind to platelet integrin receptors and to sulfatides (this work); thus, it is possible that two pools of Dab2 can be found at the platelet surface. To investigate this hypothesis, we analyzed the subcellular localization of endogenous Dab2, PTB and PTB4M in platelets activated after thrombin receptor-activating peptide (TRAP) stimulation. As expected, the endogenous Dab2 was localized peripherally after 3 min of platelet activation similarly as reported (Figure 3.4, center panel). Whereas the isolated PTB domain was clearly localized at the platelet surface after 3 min followed of TRAP stimulation (Figure 3.4, center panel), mutations in the sulfatide-binding site reduced, but did not completely abolish, the localization of the protein at the platelet surface (Figure 3.4, center panel). These results suggest that mutations in the sulfatide-binding site abrogate sulfatide binding at the platelet surface. In addition, we propose that the mutant protein can instead bind to the integrin receptor, since the PTB domain still presents the RGD (amino acids 64-66) motif, which has been shown to be responsible for binding of the PTB domain to the αIIb domain of the integrin receptor. Previous studies have shown that a Glu mutation at the residue Asp66 in the RGD site at the Dab2 PTB domain abolishes the interaction of Dab2 with the integrin receptor, without affecting lipid binding. In our hands, however, PTBD66E showed little decrease in binding to the platelet surface (Figure 3.S6, center panel).

- Binding to PtdIns(4,5)P2 mediates clathrin-dependent endocytosis of Dab2.

Interestingly, when platelets were fixed 10 min after their activation, the endogenous Dab2, its PTB domain and the PTB4M, to a lesser extent, were internalized (Figure 3.4, right panels). Platelets undergo endocytosis through two different mechanisms. One mechanism is mediated by clathrin-coated vesicles budding from specialized regions of the platelet plasma membrane called the open canalicular system, which delivers their content to α-granules. For
example, the internalization of the αIIβ3 integrin receptor has been shown to be dependent on its activation, which may be necessary to downregulate the adhesiveness of activated platelets. The second mechanism involves a clathrin-independent degradative pathway. Thus, it is possible that after signaling at the platelet surface, the Dab2 PTB domain is recycled by internalization and stored in the α-granules. Interestingly, mutation in the PtdIns(4,5)P$_2$ binding site in the PTB domain, PTB$^{K53K90}$, did not significantly affect the membrane localization of the protein (Figure 3.4, center panel), indicating that the phosphoinositide is dispensable for the localization of Dab2 at the platelet surface. However, after 10 min post-activation, platelets exhibited a 40% reduced internalization of PTB$^{K53K90}$ (Figure 3.4, right panels). This result indicates that the inability of the PTB domain to bind PtdIns(4,5)P$_2$ affects its internalization, consistent with the proposed model in which clustering of adaptor proteins, mediated by the phosphoinositide, facilitate local destabilization and membrane deformation during endocytosis.

Dab2 is implicated in receptor turnover and endocytosis. This function is mediated by its PTB domain, which interacts with receptors by their NPXY (Asn-Pro-any-Tyr) motifs. The Dab2 PTB domain depends upon the presence of phosphoinositides to initiate the clathrin-coated vesicle formation. To further investigate the mechanism by which the PTB domain itself is internalized, we have used chlorpromazine, an inhibitor that specifically blocks clathrin-mediated endocytosis. Platelets exhibited peripheral PTB domain after 3 min of their activation in the presence of the inhibitor (Figure 3.4). However, internalization of the protein was inhibited in the presence of chlorpromazine, displaying a similar phenotype to the PTB$^{K53K90}$ mutant (Figure 3.4). This observation was confirmed by incubating activated platelets with cytochalasin D, an actin polymerization inhibitor, which completely blocked Dab2 PTB domain internalization (Figure 3.4). All together, these results suggest that clathrin-coated vesicles mediate Dab2 PTB domain internalization in platelets.

- **Two pools of Dab2 exist at the surface of the activated platelet.**
  Binding of fibrinogen to platelets triggers platelet aggregation during blood clotting. It has been recently shown that the Dab2 PTB domain competes with fibrinogen for integrin binding at the platelet surface. A platelet adhesion assay was carried out using washed platelets on a plate surface in the presence of fibrinogen. To understand whether sulfatide-binding by the PTB domain is critical for integrin receptor function, we performed a competition platelet
adhesion assay. The Dab2 PTB domain did not significantly affect platelet adhesion to fibrinogen, whereas PTB\textsuperscript{4M} reduced platelet adhesion by 80% (Figure 3.5A). These results demonstrate that the sulfatide-binding mutant inhibited platelet adhesion, suggesting that abolition of sulfatide binding makes a pool of Dab2 PTB domain available to compete with fibrinogen for binding to the integrin receptor.

On the basis of our results and previously reported findings, we propose a model to describe the role of both sulfatides and PtdIns(4,5)P\textsubscript{2} in Dab2 function (Figure 3.5B). In this model, two pools of Dab2 are found at the platelet surface after its activation. One pool negatively controls platelet aggregation by competing with fibrinogen for binding to the α\textsubscript{II}β\textsubscript{3} integrin receptor. This equilibrium depends upon thrombin activation, which cleaves Dab2 at the PTB domain favoring platelet aggregation. A second pool of Dab2 is bound to sulfatides and inaccessible for thrombin cleavage. Both pools are internalized upon platelet activation by a clathrin-mediated endocytosis in a PtdIns(4,5)P\textsubscript{2}-dependent fashion.

Platelets express sulfatides on their surface, which increase after activation.\textsuperscript{27} However, the role of sulfatides during this event is still not clear.\textsuperscript{62} Whereas sulfatides activate platelets through P-selectin and enhance platelet aggregation,\textsuperscript{41} exogenous sulfatides inhibit platelet function.\textsuperscript{63} Our finding that sulfatides sequester the PTB domain, and therefore likely Dab2, away from the integrin receptor further defines the role of the lipid in platelet aggregation. In addition, our experiments indicate that internalization of the PTB domain can occur not only through α\textsubscript{IIb}β\textsubscript{3} integrin receptors, but also can be facilitated by sulfatides. This suggests an additional role of sulfatides in recycling Dab2.

Upon platelet activation, activated integrin receptors mediate localized clustering of PtdIns(4,5)P\textsubscript{2} in lipid rafts on the inner leaflet of the plasma membrane.\textsuperscript{38} Furthermore, the “flip-flop” of negatively charged phospholipids from the inner leaflet to the outer leaflet of the plasma membrane increases during the course of platelet activation.\textsuperscript{64} Activated platelets recycle integrin receptor continuously, which serves as a mechanism to downregulate the adhesiveness of platelets later in aggregation.\textsuperscript{60} Our findings suggest that Dab2 is internalized by clathrin-dependent endocytosis, and that this internalization is PtdIns(4,5)P\textsubscript{2}-dependent. Furthermore, our experiments suggest a physiological role for the competition of the two lipids for the PTB binding site. We propose that PtdIns(4,5)P\textsubscript{2} competes with sulfatides for binding to the PTB domain in order to mediate recycling of Dab2. Sulfatides drive Dab2 concentration on the surface of the activated platelet, but PtdIns(4,5)P\textsubscript{2} drives the internalization of Dab2 for recycling
into α-granules. Therefore, the two lipids balance the localization of Dab2 between the exterior and interior of the platelet. Sulfatide and PtdIns(4,5)P₂ competition for the PTB domain thus regulates Dab2’s inhibitory role in aggregation.

In conclusion, we have demonstrated that the Dab2 PTB domain specifically binds to sulfatides in a conserved site close by the one for PtdIns(4,5)P₂ ligation. While the lipid binding sites overlap through Lys53, they are distinct from each other. Furthermore, the PtdIns(4,5)P₂ competes with sulfatides for PTB binding. In platelets, sulfatide binding mediates membrane localization of Dab2 upon platelet activation. Whereas sulfatides may stabilize Dab2 during platelet activation and compete with the αIIβ3 integrin receptor for Dab2 ligation, PtdIns(4,5)P₂ participates in the internalization of the protein for its recycle and storage in α-granules. While each lipid drives a different localization of Dab2, the balanced competition between the two lipids plays a key role in platelet aggregation by regulating Dab2, an inhibitor of the αIIbβ3 integrin receptor. Perturbation of the role of sulfatides shifts the localization of Dab2 away from the surface of the platelet membrane, increasing Dab2-integrin receptor binding and ultimately decreasing platelet adhesion to fibrinogen. Disruption of PtdIns(4,5)P₂ binding, on the other hand, prevents the localization of Dab2 to α-granules in preparation for secondary aggregation. In summary, we have defined and characterized Dab2 PTB interaction with sulfatides. We have also determined the role of sulfatide and PtdIns(4,5)P₂ binding in the context of platelet aggregation. Furthermore, our findings suggest a physiological role for the competition between the two lipids, elucidating the mechanisms governing the localization of Dab2 during thrombosis. In addition, this work further defines integrin receptor regulation in the interest of therapeutic developments that specifically target the active conformation of the αIIβ3 integrin receptor using innate cellular mechanisms.
Figure 3.1: The Dab2 PTB domain interacts with sulfatides. (A) Sequence alignment of the proposed regions of the Dab2 PTB domain involved in both sulfatide and PtdIns(4,5)P₂ ligation (bold residues). Amino acids that interact with sulfatides or PtdIns(4,5)P₂ and are boxed. Consensus motifs for sulfatide binding are indicated at the bottom. (B) Nitrocellulose membranes (Sphingolipid strips) containing the indicated lipids were probed with 0.2 μg/ml GST-Dab2 PTB, according to the manufacturer’s instructions. (C) Liposome binding assay of the Dab2 PTB domain and its mutants with liposomes in the absence and presence of sulfatides. Lanes labeled with ‘S’ and ‘P’ represent proteins present in supernatants and pellets after centrifugation. GST was used as a negative control. (D) Same as C but in the absence and presence of PtdIns(4,5)P₂.
Figure 3.2: Kinetic and competitive analyses of the Dab2 PTB lipid ligands. (A) The interactions of Dab2 PTB with immobilized sulfatide (left) and PtdIns(4,5)P$_2$ (right) liposomes were analyzed by SPR detection. Resonance units indicating the bound protein fraction at increasing protein concentrations were plotted. (B) Nitrocellulose filters containing increasing amounts of sulfatides where incubated with either free or PtdIns(4,5)P$_2$-bound GST-Dab2 PTB domain. Quantification of the binding is shown on the right. (C) Competition of the lipids analyzed by SPR detection. Immobilized sulfatide liposomes were exposed to 5 µM Dab2 PTB (top) and PTB$^\text{TM}$ (bottom) with increasing concentrations of PtdIns(4,5)P$_2$ pre-incubated with the protein.
Figure 3.3: Sulfatides protect Dab2 PTB from thrombin proteolysis. (A) Ribbon (top) and surface (bottom) representation of the Dab2 PTB domain. Residues engaged in sulfatide ligation are indicated in red and yellow respectively. Lys53, a residue critical for recognition of both lipids, is labeled in orange. (B) The Dab2 PTB domain was incubated in the absence and presence of thrombin at the indicated time points and analyzed by SDS-PAGE. (C) The Dab2 PTB domain was pre-incubated with liposomes without (top) and with (bottom) sulfatides and incubated with thrombin as described in A. (D) The Dab2 PTB domain was pre-incubated with liposomes without (top) and with (bottom) PtdIns(4,5)P2 and proceeded as described in A.
Figure 3.4: Roles of sulfatides and PtdIns(4,5)P₂ in Dab2 PTB subcellular localization. Human washed platelets were incubated with Dab2 PTB, PTB⁴⁴ or PTB K⁵³K⁹⁰ domains (1.9 μM each) for 5 min at room temperature in the presence of 0.25 g/L fibrinogen (left panels). Endogenous Dab2 was also followed by the same procedure. Aggregation was initiated by the addition of TRAP at room temperature. Samples were fixed at 3 min (center panels) and 10 min (right panels) and subcellular localization of the proteins was visualized using anti-Dab2 and Cy3-coupled secondary antibodies. Quantification of the percentage of platelets showing binding and internalization of both Dab2 PTB and PTB K⁵³K⁹⁰ domains are represented by diagram bars. Scale bar is 5 μm.
**Figure 3.5:** Two pools of Dab2 likely exist at the activated platelet surface. 

(A) Human washed platelets were activated in fibrinogen-coated wells in the presence of Dab2 PTB or PTB\textsuperscript{4M} proteins (1.9 \(\mu\)M), fixed, and stained with Wright stain. Stain was eluted with 20\% ethanol and quantified at 415 nm. (B) Model showing two pools of Dab2 at the activated platelet surface (integrin receptor bound protein and sulfatide bound protein) and the PtdIns\(4,5\)P\(_2\) mediated endocytosis of Dab2.
Figure 3.S1: PTB-sulfatide interaction requires residues from both conserved basic motifs. (A) Nitrocellulose membranes containing the indicated pmoles of sulfatides were probed with 1 µg/ml GST or GST-PTB constructs. (B) Liposome binding assay of Dab2 PTB mutants in the absence or presence of sulfatides. Lanes labeled ‘S’ and ‘P’ represent proteins present in supernatants and pellets after centrifugation.

Figure 3.S2: Mutations do not alter the secondary structure of Dab2 PTB. Circular dichroism (CD) was performed with 5 µM PTB constructs. Spectra were converted to mean residue ellipticity using DICHROWEB and deconvoluted using CDSSTR.
Table 3.1: Secondary structure composition of Dab2 PTB. Predictions were generated using DICHROWEB and deconvoluted using CDSSTR.

<table>
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**Figure 3.33:** Mutations in the sulfatide binding sites do not significantly alter PtdIns(4,5)P₂ binding. Liposome binding assay of Dab2 PTB mutants in the absence or presence of sulfatides. Lanes labeled ‘S’ and ‘P’ represent proteins in supernatant and pellet fractions after centrifugation.

**Figure 3.34:** Sulfatide-phosphoinositide competition for Dab2 PTB binding does not occur nonspecifically. GST-Dab2 PTB was pre-incubated in the absence and presence of 10-fold excess of PtdIns(3)P and further incubated with nitrocellulose membranes containing increasing amounts of sulfatides. GST was used as a negative control. Quantification of the binding is shown on the right.
**Figure 3.S5:** Controls for immunofluorescence analysis. Human washed platelets were incubated with Bovine Serum Albumin (BSA) control (1.9 μM), dimethyl sulfoxide (DMSO), or H₂O (vehicle controls) for 5 min at room temperature in the presence of 0.25 g/L fibrinogen (left panels). Aggregation was initiated by the addition of TRAP at room temperature. Samples were fixed at 3 min (center panels) and 10 min (right panels) and subcellular localization of the proteins was visualized using anti-Dab2 and Cy3-coupled secondary antibodies. Scale bar is 5 μm.

**Figure 3.S6:** Dab2 PTB<sup>D66E</sup> shows little decrease in binding and internalization in activated platelets. Human washed platelets were incubated with Dab2 PTB<sup>D66E</sup> for 5 min at room temperature in the presence of 0.25 g/L fibrinogen (left panels). Aggregation was initiated by the addition of TRAP at room temperature. Samples were fixed at 3 min (center panels) and 10 min (right panels) and subcellular localization of the proteins was visualized using anti-Dab2 and Cy3-coupled secondary antibodies. Scale bar is 5 μm.
Chapter 4: Summary and Conclusions

In summary, we have characterized the biochemical mechanisms and kinetics of the interaction of a novel lipid ligand, sulfatides, with the lipid binding pocket of the Dab2 PTB domain. We have also defined the mode of interaction of sulfatides with the known lipid ligand, PtdIns(4,5)P\textsubscript{2}, for binding to the PTB domain. Finally, we demonstrated the physiological relevance of PTB-sulfatide binding in the context of platelet aggregation and platelet endocytosis. Our work has defined a novel regulatory mechanism of Dab2, which in turn inhibits the α\textsubscript{IIb}β\textsubscript{3} integrin receptor. Understanding the lipid-mediated control of Dab2 will contribute to the growing pursuit of anti-platelet prophylactics that target the integrin receptor to prevent acute thrombosis.

Our research has determined the residues that mediate PTB-sulfatide versus PTB-PtdIns(4,5)P\textsubscript{2} ligand interaction. We used site-directed mutagenesis to demonstrate that lysine residues 25, 49, 51, and 53 specifically mediate sulfatide binding (Figure 3.1). We have also shown that the binding site for PtdIns(4,5)P\textsubscript{2} is distinct from the sulfatide binding region of the PTB lipid binding pocket, although both binding sites share Lys53 (Figure 3.S1, Figure 3.S3). In addition, our data suggest that the two lipids compete for PTB binding (Figure 3.2).

In vivo, we have defined the mechanism by which sulfatides govern the stability, localization, and functional role of Dab2 during platelet aggregation. First, we demonstrated that sulfatide binding, unlike PtdIns(4,5)P\textsubscript{2} binding, protects the PTB domain from cleavage by thrombin, a potent platelet activator in the coagulation cascade (Figure 3.3). Next, we visualized the localization of endogenous Dab2 during platelet aggregation, showing that the protein is localized on the outer periphery after activation and is ultimately internalized at 10 minutes after activation (Figure 3.4). Using exogenously added PTB protein, we have shown that abrogation of sulfatide binding significantly reduces, but does not completely abolish, peripheral localization of Dab2 after activation (Figure 3.4). Our data suggest a role for sulfatide-PTB binding to drive Dab2 localization at the activated platelet surface. Furthermore, we demonstrated that PTB-PtdIns(4,5)P\textsubscript{2} binding is necessary for internalization of the Dab2 PTB after activation. By utilizing inhibitors of actin reorganization and clathrin coat assembly, we have shown that Dab2 PTB internalization is driven by clathrin-dependent endocytosis. Therefore, both PTB lipid ligands play a distinct role in governing the equilibrium between the intracellular and extracellular localization of Dab2 at the surface of the activated platelet.
Finally, we addressed the impact of Dab2-sulfatide binding on platelet function. Our data indicate that two pools of Dab2 likely exist at the activated platelet surface: one pool bound to sulfatide lipids and the second pool bound to the αIIbβ3 integrin receptor (Figure 3.5). Abolition of sulfatide binding shifts the two pools to favor integrin receptor binding, thus enhancing Dab2’s negative regulation of integrin-fibrinogen binding. Thus, Dab2-sulfatide binding balances Dab2-mediated inhibition of platelet aggregation by modulating the two pools of Dab2 at the activated platelet surface.

In the broader picture of therapeutic relevance, our work has defined a negative regulatory mechanism of the αIIbβ3 integrin receptor. The αIIbβ3 integrin receptor is the most prevalent receptor on the platelet surface and ultimately drives platelet aggregation by binding to fibrinogen. Although anti-platelet drugs target the αIIbβ3 integrin receptor to prevent acute arterial thrombosis, these approaches have high incidences of bleeding disorders and accidental activation of the integrin receptor. Our work has defined a novel regulatory mechanism of Dab2, an affinity-state specific regulator of the αIIbβ3 integrin receptor. Prophylactic use of activation-specific integrin regulators represents a prominent direction of anti-platelet research. Defining the mechanisms that govern the integrin receptor will facilitate the development of anti-integrin therapeutics utilizing inherent cellular mechanisms.
Chapter 5: Materials and Methods

Chemicals. The following is a list of chemicals used and their suppliers: brain sulfatides, dipalmitoyl phosphatidylcholine (PC), dipalmitoyl phosphatidylethanolamine (PE), dipalmitoyl phosphatidylserine (PS) (Avanti Polar Lipids), cholesterol (Sigma), dipalmitoyl and dioctanoyl PtdIns(4,5)P2 (Cayman Chemicals). All other chemicals were analytical reagent grade.

DNA cloning, plasmids and protein expression and purification. Flag-tagged full-length human Dab2 cDNA construct was cloned into a pCS2+MT vector. The Dab2 PTB domain (residues 1-241) cDNA construct was cloned into a pGEX6P1 vector (GE Healthcare). Site directed mutagenesis of Dab2 and its PTB domain were performed using the Quick-Change exchange protocol (Stratagene). Expression and purification of all GST-fusion proteins from E.coli Rosetta cells (Novagen) on glutathione beads was performed as previously described.65 Purity of all proteins was over 95% as judged by SDS-PAGE gels.

Protein-lipid overlay assay. Membrane strips (SphingoStrips™) spotted with 100 pmol of sphingolipids were purchased from Echelon Research Laboratories. Membrane strips were incubated with 0.1 μg/ml of the Dab2 PTB domain or its mutants in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Tween-20 and 3% fatty acid-free bovine serum albumin overnight at 4°C. Following four washes with the same buffer, proteins bound to the membrane strips were probed with rabbit anti-GST antibody (Santa Cruz Biotech). Donkey anti rabbit-horse radish peroxidase (HRP) antibody was obtained from GE Healthcare. Detection was carried out using ECL reagent (Pierce). Also, lipid strips were prepared by spotting 1 μl of either sulfatides or PtdIns(4,5)P2 dissolved in chloroform:methanol:water (1:2:0.8 and 65:35:8, respectively) onto Hybond-C extra membranes (GE Healthcare) and protein binding was monitored as described above. Bound protein was quantified using the Alphalmager program and binding was calculated as a percentage of GST-PTB binding to 100 pmoles of spotted lipid.

Liposome-binding assay. Lipid stocks including sulfatides, PE, PS, and PtdIns(4,5)P2 were dissolved in chloroform:methanol:water (1:2;0.8, 65:35:8, 65:35:4 and 65:35:8, respectively), whereas cholesterol and PC were dissolved in chloroform:methanol (1:1). Sulfatides liposomes were prepared in a weight ratio of 1:1:1:4 of PC:PE:cholesterol:sulfatides. PtdIns(4,5)P2 liposomes were prepared in a percent ratio of 50:20:10:10:10 of PC:PE:PS:cholesterol:PtdIns(4,5)P2. Controls were prepared by adjusting the ratios with PC. Lipid films were generated by lyophilization overnight and hydrated in 20 mM Tris-HCl (pH
6.8), 100 mM NaCl, 2 mM dithiothreitol (DTT) at 1 mg/mL and freeze-thawed 3 times. Liposomes were sonicated and further pelleted and suspended in 4 mg/mL. Ten μg of protein were incubated with 125 μg of total lipids for 20 min at RT. Liposome-bound and free-protein samples were separated by centrifugation and analyzed by SDS-PAGE. Bands were quantified using AlphaImager, and the percentage of total protein each fraction contained was calculated from the total protein.

**Surface plasmon resonance.** Surface plasmon resonance (SPR) binding experiments were performed on a BIACore X instrument using L1 sensorchips coated with ~0.5 mM of mixed sulfatides or PtdIns(4,5)P₂ size calibrated liposomes. Dab2 PTB domain or its mutants binding experiments were performed in a degassed solution containing 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl. This buffer was used during equilibration, association and dissociation phases. Proteins were added to this buffer at the indicated concentrations. Regeneration of the phospholipid bilayer after dissociation phase was carried out using 20 mM NaOH. For competition SPR, increasing molar amounts of dioctanoyl PtdIns(4,5)P₂ were preincubated with 5μM Dab2 PTB for 20 min at room temperature. The entire reaction was then exposed to an L1 sensorchip containing immobilized sulfatide liposomes (~0.5 mM).

**Thrombin limited proteolysis.** Sulfatides- and PtdIns(4,5)P₂-enriched liposomes were incubated with 10 μg of protein for 30 min at room temperature, the reaction centrifuged, and pellets suspended in 50 μl of liposome binding buffer. Thrombin (0.05 units/μg protein) was added to both supernatants and pellets, and aliquots were taken at 0, 8, 12, 16, and 20 h after digestion. Reactions were stopped by the addition of Laemmli buffer and analyzed by SDS-PAGE.

**Platelet immunofluorescence analysis.** Washed platelets (50 μl of 3x10⁻⁸ platelets/mL in Tyrode's albumin buffer) were incubated with 1.9 μM of PTB domain constructs for 5 min at room temperature in the presence of 0.25 g/L fibrinogen. Aggregation was initiated by the addition of 10 μM TRAP. Reactions were incubated at room temperature unless otherwise indicated. Platelets were fixed with 0.4 mL of 3.7% formaldehyde in Phosphate Buffered Saline (PBS) for 30 min. Next, 20% goat serum was added to the fixed platelets, and 60% of the total fixed reaction was centrifuged using a Shandon cytopspinner onto a Shandon coated cytoslide to achieve the appropriate platelet density for analysis. Slides were then washed twice with PBS for 10 min. Platelets were permeabilized with 0.5% Triton X-100 in PBS for 10 min and then
blocked with 20% goat serum in PBS with 0.1% Triton X-100 for 1 h at room temperature. Platelets were washed 3 times with PBS with 0.1% Triton for 10 minutes and further incubated overnight with the anti-Dab2 antibody 1:100 (anti-p96; BD Transduction) in PBS with 0.1% Triton X-100 and 10% goat serum at 4°C in a humidity chamber. Platelets were then washed and incubated with the anti-mouse Cy-3 conjugated goat antibody 1:250 and Alexa488 conjugated phalloidin (25 µg/mL) in PBS with 0.1% Triton X-100 and 10% goat serum for 2 h. Platelets were washed again and observed on an Olympus IX71 microscope using the Softworx Applied Precision software.

Mean pixel intensity was determined for 400 platelets from four fields each for 3 min and 10 min of treatments by manually drawing circles around individual platelets using the 2D Polygon tool in the Softworx software. For each time point, a threshold of significant mean pixel intensity was defined for the experiment. The percentage of countable platelets to exceed the threshold was calculated for each field and the average of four fields was computed with standard deviation. The ratio of internalization:binding is the quotient of these averages for a treatment.

**Platelet adhesion assay.** Human washed platelets were incubated with BSA, Dab2 PTB or Dab2 PTB\(^{TM}\) (1.9 µM each) for 5 min at room temperature in the presence of 0.25 g/L fibrinogen. Aggregation was initiated by the addition of 10 µM TRAP, and reactions were incubated 20 min at 37°C. Samples were fixed with 3.7% formaldehyde in PBS, stained with Wright stain, and photographed. Wright stain was eluted with 20% ethanol, and absorbance was quantified at 415 nm.

**CD spectroscopy.** Spectra were collected using a Jasco J-720 spectropolarimeter. Far-UV CD spectra were measured for Dab2 PTB constructs (5µM in 5mM Tris, 100 mM potassium fluoride, 100 µM DTT, pH 6.8) using a 1mm-slit-width cuvette. Five accumulated scans were recorded for each sample between 190 and 260 nm at 20°C in increments of 0.5 nm. Background of buffer alone was subtracted from each spectrum. Raw data were converted to mean residue ellipticity and analyzed for secondary structure composition using DICHROWEB and deconvoluted using CDSSTR.
Chapter 6: Collaborations

A. **Apoptosis in *Xenopus* embryos is preferentially triggered by short bursts of energetic x-rays.** Jiajia Dong, Karen E. Drahos, Royce K. Zia, Carla V. Finkielstein. *In preparation.*

Gamma-irradiation induces DNA breaks, ultimately resulting in apoptosis in cells. The total amount of radiation administered is a product of the energy of incident photons (voltage) and duration of radiation (time). Using *Xenopus laevis* embryos, we have demonstrated that a threshold of incident photon energy exists. When irradiated at a voltage below this threshold, the embryos survive; however, when given the same total dose of irradiation at a higher incident photon energy, the embryos die by apoptosis. We have used cyclin A2 cleavage assays, caspase cleavage assays, and Terminal Deoxynucleotidyl Transferase dUTP nick end labeling to demonstrate that the total dosage of radiation is not what induces apoptosis; instead, the energy of the incident photons determines whether cells will undergo apoptosis. I contributed Figure 2_Dong et al. and Figure 6_Dong et al. to this project. All of the authors contributed to the intellectual development of the project.
Figure Legends

Figure 1. The energy-dependent hypothesis of apoptosis induction. A. Schematic representation of a typical dose dependency curve. Here, we show how the total dose $E$ is a combination of the energy of each photon ($h\nu$) and the time exposed ($T$), assuming that the current ($j$) remains fixed. Three different scenarios are denoted: (A) $E$ is variable and results from increasing the energy of the beam while maintaining $T$ constant; (B) increase $E$ by increasing $T$ alone, without changing $h\nu$. If this event occurs below the threshold (dotted line), no electrons will be emitted independently of the exposure time; (C) $E$ remains constant throughout the states analyzed and results from a proportional combination of the energy of the photon and exposure time. Each color line represents a different total dose. B. Summary of the experimental conditions to be analyzed in order to evaluate the scenarios discussed above.

Figure 2. Apoptosis results from exposure to increased energies. A. Embryos were irradiated ($\gamma$-IR) or not (control) before the MBT (st.6) with the indicated amount of energy (<10 kV, 10 kV, 20 kV, 30 kV, 40 kV, 50 kV) for 12 min, collected at st.8 (MBT) and 4, 6 and 8 h after the MBT, and frozen. At the indicated times, samples equivalent to ten embryos were tested for the activity of caspases 3/7 using a specific colorimetric substrate as described in the “Materials and Methods” section. Normalized caspase activity refers to the activity of irradiated samples from which the basal control activity has been subtracted at each time and is expressed in relative units (RU). The figure shows data from a single experiment that was repeated three times with similar results. Dotted line denotes a threshold of caspase activity from which experimental values falling below correlate with embryo samples lacking of an apoptotic response. B. Extracts equivalent to ten embryos collected at the indicated times were incubated with radiolabeled cyclin A2 as described in the “Materials and Methods” section. At the indicated times (0 and 120 min), aliquots were removed and analyzed for cyclin A2 cleavage by SDS-PAGE and autoradiography. Control samples correspond to non-irradiated embryos. Arrows on the right denote radiolabeled Xenopus cyclin A2 (XA2) and its cleaved form. Molecular mass markers (in kDa) are indicated on the left.

Figure 3. A minimum energy value is required to trigger apoptosis. Embryos were irradiated ($\gamma$-IR) or not (control) before the MBT (st.6) with either 20 kV (A), 30 kV (B), 40 kV (C), 50 kV (D) or 60 kV (E) of energy for the indicated times, collected at st.8 (MBT) and 4, 6 and 8 h after the MBT, and frozen. Samples equivalent to ten embryos were tested for caspases 3/7 activity using a specific colorimetric substrate as described in the “Materials and Methods” section and normalized as described in the legend of Figure 2. Results similar to those presented here were observed in three independent experiments.

Figure 4. High-dose radiation raises caspase activity and favors cyclin A2 cleavage. Extracts equivalent to ten embryos from non-irradiated (control) or irradiated ($\gamma$-IR) samples collected at MBT (st.8) and 4 h after the MBT were incubated with radiolabeled cyclin A2 as described in the “Materials and Methods” section. Aliquots were removed at the indicated times and analyzed for cyclin A2 cleavage by SDS-PAGE and autoradiography. Control samples correspond to non-irradiated embryos. Arrows on the right denote radiolabeled Xenopus cyclin A2 (XA2) and its cleaved form.
**Figure 5.** *Xenopus* embryos exhibit different biological responses to the same dose of radiation. A. Samples equivalent to ten embryos irradiated (\(\gamma\)-IR) or not (control) before the MBT were collected at the indicated times and assayed for caspase activity as described in the legend of Figure 2. The figure shows data from a single experiment that was repeated three times with similar results. B. Caspase 3/7 activity was also assessed by cleavage of the radiolabeled cyclin A2 in extracts form control (non-irradiated) or \(\gamma\)-IR with the same total dose as indicated. Arrows on the right denote radiolabeled *Xenopus* cyclin A2 (XA2) and its cleaved form.

**Figure 6.** Whole-mount TUNEL assay exposes apoptotic cells in irradiated embryos. A. Pre-MBT embryos were exposed to different energies (20 kV, 30 kV, 40 kV and 48 kV) for the indicated times (24 min, 16 min, 12 min, 10 min) to equal a total dose of 48 Gys. Non-irradiated embryos are referred as “control”. Eight hours after the MBT, embryos were fixed in MEMFA as described in the “Materials and Methods” section and photographed. B. TUNEL staining was performed on fixed embryos to detect DNA fragmentation. Embryos were treated as described in (A). Intense TUNEL staining was detected in the animal pole portion of the embryos. The embryos shown in B are representative of the TUNEL staining observed following analysis of ~80 embryos of which 20% were stained.

**Figure 7.** The energy model of the apoptotic response. A. Schematic representation of the dose dependency curve where all the experimental conditions are plotted. Arrows indicate each of the three scenarios tested. Energy was measured in kilovolts (kV) and time (T) in min. Color lines indicate the same total dose. Symbols indicate various energies tested: ◆: <10 kV, ■: 10 kV, ○: 20 kV, ◇: 30 kV, □: 40 kV, ●: 50 kV, △: 60kV. B. Conceptual model for the contribution of energy and exposure time to the induction of apoptosis. Energy (kV) is delivered to the sample in either small (left) or large (right) quantum packages (where small packages are represented as \(\frac{1}{4}\) of the larger packages) for a period of time \(t\) where \(t_2=3t_1\). In our model, apoptosis is exclusively induced when larger packages of energy are delivered to the sample even when the total exposure dose is the same in both scenarios.
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![Voltage-Time graph]

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![Graph showing the normalized Caspase 3/7 activity over different embryo stages and voltages.](image)

B. 

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Figure 4 Dong et al.
Figure_5 Dong et al.

A. Normalized Caspase 3/7 Activity (RU)

B. 

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Figure 7 Dong et al.

B.

Apoptosis

Apoptosis

Contributions I have made to this project are stated in the Acknowledgements section of the publication (see attached):

“…K.E.D., C.S.S., and S.P.M. performed the experiments described in the supplemental material and provided technical support at various stages of the project…..”
A Novel Heme-Regulatory Motif Mediates Heme-Dependent Degradation of the Circadian Factor Period 2

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Although efforts have been made to identify circadian-controlled genes regulating cell cycle progression and cell death, little is known about the metabolic signals modulating circadian regulation of gene expression. We identify heme, an iron-containing prosthetic group, as a regulatory ligand controlling human Period-2 (hPer2) stability. Furthermore, we define a novel heme-regulatory motif within the C terminus of hPer2 (SC841PA) as necessary for heme binding and protein destabilization. Spectroscopy reveals that whereas the PAS domain binds to both the ferric and ferrous forms of heme, SC841PA binds exclusively to ferric heme, thus acting as a redox sensor. Consequently, binding prevents hPer2 from interacting with its stabilizing counterpart cryp-tochrome. In vivo, hPer2 downregulation is suppressed by inhibitors of heme synthesis or proteasome activity, while SA841PA is sufficient to stabilize hPer2 in transfected cells. Moreover, heme binding to the SC841PA motif directly impacts circadian gene expression, resulting in altered period length. Overall, the data support a model where heme-mediated oxidation triggers hPer2 degradation, thus controlling heterodimerization and ultimately gene transcription.

Cellular homeostasis depends on a delicate balance between metabolic activity and gene expression. Heme is a prosthetic group essential for transport and storage of oxygen that is involved in the generation of cellular energy by respiration and synthesis and degradation of lipids and in oxidative damage. Heme-based sensor proteins detect and respond to variations in oxygen, carbon monoxide, and nitric oxide levels and cellular redox state by acting on transcription, translation, protein translocation, and protein assembly (8, 25).

Heme binding to transcription factors is found in both prokaryotes and lower eukaryotes; however, only three cases have been identified in higher eukaryotes: the basic leucine zipper transcription factor Bach1, the circadian transcription factor NPAS2, and the nuclear orphan receptor Rev-erbs (6, 23, 30, 43). Although structurally unrelated, both NPAS2 and Bach1 regulate the transcription of genes impacting heme synthesis and degradation (15, 36). Heme binds to NPAS2 through a region of homology called PAS (Per-ARNT-Sim) to form a gas-regulated sensor (6). Although DNA binding of NPAS2 depends on its heterodimerization with another basic-helix-loop-helix-PAS protein termed Bmal1, heme is not required for the dimer to bind DNA (6). Heme-bound NPAS2 acts as a gas-sensing protein by binding carbon monoxide, causing inhibition of the DNA-binding capacity of NPAS2/Bmal1 (6). Interestingly, heme binding to Rev-erbs is mediated by a histidine residue located in the carboxy tail of the ligand-binding domain, but unlike NPAS2, Rev-erbs activity is not responsive to diatomic gases and is unlikely to sense redox conditions (30, 43).

Along with PAS, a second domain has been identified as a heme-binding site. The heme-regulatory motif (HRM) comprises a stretch of residues where only a Cys-Pro core is absolutely conserved and a preferred hydrophobic residue is located in the fourth position (X-CP-b). This motif has been identified in functionally diverse proteins and is thought to govern the activity of a neighboring transmitter domain in response to heme binding (45). For example, heme binds to the transcriptional repressor of the heme oxygenase-1 (HO-1) gene, Bach1, through multiple HRMs (37). Binding inhibits Bach1/MafK association with the HO-1 promoter, inducing subcellular relocalization of Bach1 and degradation (37, 44). In addition to Bach1, various heme-mediated protein functions require HRMs: the yeast transcriptional activator Hap1 that transcribes genes encoding various cytochromes, catalase, and Rox1, which represses anaerobic genes under high heme concentration (see reference 12 and references within); the heme-regulated inhibitor kinase that controls the activity of the translation initiator factor elf-2α in stressed erythroid cells (4, 11); the erythroid 5-aminolevulinic acid synthase precursors whose transport to the mitochondria is mediated by heme binding to HRMs (20); the heme lyase found in both Saccharomyces cerevisiae and Neurospora crassa (35); the mammalian nuclear factor erythroid 2 that plays a critical role in erythroid differentiation (22); the HO-2 that metabolizes heme (21); the iron regulatory protein 2 (IRP2), a regulator of iron metabolism in mammals (13), and the iron response regulator (Irr) in bacteria whose turnover depends on the cellular iron availability (28, 42).

A second PAS-containing circadian molecule, Per2, has also been implicated in heme binding and mediates per1 and per2 transcription in vivo by a mechanism involving NPAS2 (15).
Disruption of either the per1 or per2 gene in mice leads to circadian deregulation of heme biosynthesis by altering the expression levels of the rate-limiting enzymes Alas1 and Alas2 (15, 46). Unlike Npas2, Per2 does not contain a basic-helix-loop-helix domain, and it is hypothesized that heme control of Per2-mediated gene transcription takes place indirectly by modulating the expression of Bmal1. Consequently, while we know much about how heme and Per2 signaling molecules operate in cell metabolism and circadian rhythms, we lack a clear understanding of how these two circuitries are integrated and operate to directly modulate gene expression.

Here we report the discovery of a previously uncharacterized heme-regulatory motif in Per2 with a functional link to protein stability. We show that (i) heme binds to two distinct regions of human Period-2 (hPer2) and the oxidation state of the heme iron determines binding specificity and degradation; (ii) hPer2 stability is compromised when heme binds to the outermost C-terminal domain of the protein, preventing hPer2 from binding its heterodimeric counterpart human cryptochrome 1 (hCry1); (iii) downregulation of hPer2 is suppressed in the presence of heme synthesis or proteasome activity; and (iv) a point mutation in the C-terminal HRM is sufficient to stabilize hPer2 in vivo. Together, our data indicate that an uncharacterized HRM functions as a binding site and triggers heme-induced degradation of hPer2, likely regulating cellular signaling by modulating the formation of hPer2/hCry1 complex.

MATERIALS AND METHODS

Plasmid constructs and site-directed mutagenesis. Various hPer2 and hCry1 cDNA fragments were cloned into the SalI and NotI sites of pGEX-4T-3. Fragments of hPer2 comprising residues 1 to 172, 173 to 355, 356 to 574, 173 to 574, hPer2(II-III), hPer2(V4), and hPer2(V4-VII), respectively. The Cys residue of each putative HRM (Cys\(^611\) and Cys\(^626\)) and Cys\(^626\) in hPer2 was mutated to Ala by site-directed mutagenesis using QuikChange (Stratagene). The hPer2, hPer2(\(^{SA}^{841}PA\)), hPer2(II-III), and hPer2(V4-VII) cDNAs were cloned into pCS2\(^{-}\)/myc-tag vector modified for ligation-independent cloning (Novagen).

Protein pull-down and heme-agarose-binding assays. Glutathione S-transferase (GST) fusion proteins were expressed in Escherichia coli strain Rosetta (Novagen) and purified by glutathione-Sepharose chromatography following the manufacturer's instructions (GE HealthSciences). Untagged proteins were generated by digestion of fusion proteins with thrombin followed by concentration and buffer exchange (10 mM Tris-HCl [pH 8.0]). For pull-down assays, a total of 5 \(\mu\)g of GST-hCry1-bound beads or an equivalent amount of glutathione beads were added to 5 \(\mu\)g of either hPer2 or the preformed GST-hCry1/hPer2 complex at 4°C for 1 h. After the beads were washed with low- and high-salt binding buffer A (with 100 mM NaCl and 1 M NaCl, respectively), bound proteins were eluted by boiling in Laemmli sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. In other experiments, heme (Fe[III]-heme, 10 \(\mu\)M) was added to either hPer2 or the preformed GST-hCry1/hPer2 complex and incubated at 4°C for 1 h. In the first scenario, hPer2 heme was loaded onto GST-hCry1 beads, and binding proceeded at 4°C for an additional hour. Samples were analyzed by autoradiography.

For heme-agarose binding, 20 \(\mu\)l of heme-agarose beads (Sigma) was washed, resuspended in binding buffer B (10 mM sodium phosphate buffer [pH 7.5], 500 mM NaCl, 5 mM EDTA, 1% Triton X-100) and incubated with 5 \(\mu\)g of the indicated recombinant proteins at 4°C for 1 h. Beads were washed with low- and high-salt binding buffer B (with 250 mM and 1 M NaCl, respectively), and proteins were analyzed by SDS-PAGE.

Spectroscopic analysis of heme-protein binding. Ferric heme binding was determined by absorption spectra of 1 \(\mu\)M heme in the absence or presence of 1 \(\mu\)M of indicated proteins in 10 mM Tris-HCl, pH 8.0. The protein/hemin molar ratio ranged from 0.25 to 8. Results were plotted as absorbance at the peak versus the molar ratio of protein to heme. To determine ferrous heme-binding properties, 30 mM sodium dithionite was added to reduce heme to ferrous heme. Absorption spectra were recorded between 300 and 700 nm on a Beckman DU-640 UV-visible spectrophotometer.

CD spectroscopy. Far-UV circular dichroism (CD) spectra were measured on a Jasco J-720 spectropolarimeter using a 1-mm-slit-width cuvette. The hPer2(V4-VII) protein (8.3 \(\mu\)M) was titrated against increasing concentrations of heme (molar protein/heme ratios of 1:1, 1:2, and 1:4) in 10 mM phosphate buffer (pH 7.6) and 150 mM NaCl. Five accumulated scans for each sample were recorded from 190 to 240 nm with an increment of 0.5 nm, a scan rate of 50 nm min\(^{-1}\), a response time of 4 s, and a sensitivity of 50 millidegrees at room temperature. All CD spectra were corrected by subtraction of the background from the spectrum obtained with either buffer alone or buffer containing heme. Raw data were converted to mean residue ellipticity, \(\theta\), in degrees cm\(^2\) dmol\(^{-1}\). A similar procedure was followed for hPer2(II-III) and hPer2(V4-VII)\(^{SA}^{841}PA\). Data were analyzed for protein secondary structure using DICHROWEB (38) and deconvoluted using DCDSTR (34).

In vitro degradation assays. For protein degradation experiments, Chinese hamster ovary (CHO) cell extracts were prepared in lysis buffer (Promega) containing 25 mM Tris-HCl (pH 7.8), 2 mM EDTA, 2 mM dithiothreitol (DTT), 10% glycerol, and 1% Triton X-100. Alternatively, commercially available HeLa cell extracts (fraction S100 from Biozol) were also used in these experiments. For in vitro degradation assays, \(^{35}\)S-labeled fragments of Cry1, Mdm2, hPer2, hPer2(\(^{SA}^{841}PA\)), and hPer2 proteins were incubated with cell extracts at 37°C supplemented with ubiquitin (0.1 mg/ml) and an energy-regenerating system. Hemin was added to the mixture to a final concentration of 10, 25, 50, or 100 \(\mu\)M. Reactions were stopped by the addition of Laemmli sample buffer, resolved by SDS-PAGE, and visualized by autoradiography. Denaturation quantification was carried out using a FluorChem digital imaging system (Alpha Innotech).

Cell culture and analysis of endogenous Per2 protein. CHO cells were maintained in F-12K medium (Invitrogen) supplemented with 10% fetal bovine serum and gentamicin (50 \(\mu\)g/ml). To detect endogenous levels of Per2, cells were cultured in serum-free medium containing 5 mM succinylacetone for 24 h prior to heme addition (10 \(\mu\)M). Cells were harvested at the indicated times after treatment, and pellets were resuspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 10 mM MgCl\(_2\), 200 mM NaCl, 1% NP-40, 5% glycerol). For detection of hCry1 levels, the procedure was essentially the same as the one described above except that cells were first transfected with pCS2\(^{-}\)/myc-hCry1 using Lipofectamine and cultured for 12 h before the addition of succinylacetone. Endogenous Per2 and myc-hCry1 levels were detected by immunoblotting using specific antibodies (Santa Cruz).

Shock procedures and sample collection. Low-density CHO cells were plated 4 days before the experiment, transfected with 0.5 \(\mu\)g of pCS2\(^{-}\)/myc-hPer2 or hPer2(\(^{SA}^{841}PA\)) using Lipofectamine and cultured for 12 h before synchronization (1). Briefly, at time zero, the medium was exchanged with 50% F-12K medium supplemented with 50% horse serum and gentamicin (50 \(\mu\)g/ml). After 2 h of incubation, cells were washed twice with phosphate-buffered saline (PBS), and the medium was replaced with serum-free F-12K medium containing 5 mM succinylacetone. Hemin (10 \(\mu\)M) was added 24 h after serum shock, and the cells were maintained for 6 h before the medium was replaced with serum-free F-12K medium containing 5 mM succinylacetone. At the indicated times, cells were washed with PBS, frozen, and kept at −80°C until the extraction of whole-cell RNA. Reverse transcription-PCRs were performed using specific primers for Rex-erba and GAPDH (glyceraldehyde-3-phosphate dehydrogenase gene) (see supplemental material for details).

Cell transfection and immunofluorescence assays. CHO cells were cultured on coverslips for 24 h. Cells were then transfected with 0.5 \(\mu\)g of pCS2\(^{-}\)/myc-hPer2 or hPer2(\(^{SA}^{841}PA\)) using Lipofectamine (Invitrogen) and cultured for an additional 12 h. The effects of heme on myc-hPer2 and hPer2(\(^{SA}^{841}PA\)) levels were determined using transfected cells treated with either 10 \(\mu\)M heme or solvent for 2 h. After incubation, cells were maintained in serum-free medium for an additional 6 h and fixed in 3.7% formaldehyde-PBS-0.5% Triton X-100 at room temperature. Fixed cells were washed with PBS containing 0.5% Triton X-100 and then 0.1% Triton X-100 and blocked with goat serum at room temperature for 30 min. For cellular localization of myc fusion proteins, cells were transfected using a Cy3-conjugated anti-myc antibody (Sigma). Nuclei were detected by incubating fixed cells with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes). Fluorescence was visualized using a DeltaVision Core microscope equipped with a CoolSnap HQ2 camera (Applied Precision) at 457 nm, 528 nm, and 617 nm. Signal intensities were measured using the profile plot analysis.
RESULTS

Heme regulates hPer2 stability. Like other cellular pathways, the circadian clock relies on mechanisms of synthesis and degradation of some of its components to sustain oscillations. Heme stimulates the expression of transcription factors that regulate circadian rhythms by modulating the activity of the Bmal1/NPAS2 complex, which transcriptionally controls the expression of the mammalian period genes and of the alias1 gene (6, 15). Because there is little evidence regarding the mode by which heme acts on eukaryotic circadian transcription factors, we aimed to elucidate the molecular basis by which heme binding influences hPer2 function. First, we monitored the degradation of radiolabeled hPer2 in a cell-free system in response to heme [Fe(III)]-heme] treatment. 35S-labeled hPer2 was incubated with a cell extract as the source for ubiquitination enzymes and proteasome in the presence of various concentrations of heme. Results show hPer2, but not a non-specific control protein (Mdm2 [see Fig. S1 in the supplemental material]), is degraded shortly after the addition of hemin in a dose-dependent manner (Fig. 1A). Incubation with 1, 10, and 100 µM of ligand resulted in a rapid reduction (~20, 60, and 90%, respectively) of hPer2 levels (Fig. 1A and data not shown). Importantly, this effect was inhibited when cell extracts were preincubated with the proteasome inhibitor MG-132, suggesting that heme-dependent degradation of hPer2 is mediated by the ubiquitin-proteasome pathway (Fig. 1A). Next, we investigated whether heme binding to the hPer2 PAS domain mediates hPer2 turnover. Interestingly, 35S-labeled hPer2(II-III) (residues 173 to 574, comprises the PAS domain) remained stable in a cell-free assay even at high hemin concentrations (Fig. 1B), suggesting that regions other than PAS contain heme-regulated instability elements mediating hPer2 degradation.

Casein kinase epsilon (CKIε), a central component of the mammalian circadian clock, is the prime kinase involved in hPer2 downregulation by direct targeting of Ser662 for phosphorylation (3, 7). To rule out any contribution of CKIε to heme-mediated degradation of hPer2, we analyzed 35S-labeled hPer2(S662A) in a cell-free system for its stability in the presence of heme (Fig. 1C). hPer2 levels remained stable in the absence of heme in cell extracts, ruling out the contribution of other phosphorylation events in hPer2 stability. Results indicate that hPer2(S662A) levels remain sensitive to heme addition, supporting the existence of a novel mechanism for hPer2 degradation that is independent of CKIε phosphorylation but dependent on the presence of heme.

Because hPer2 can be efficiently degraded in vitro, we were prompted to look for evidence of heme-mediated degradation in vivo. First, endogenous Per2 levels were monitored in CHO cells after heme addition. Time course experiments showed reduced levels of Per2 protein but not its mRNA upon incubation with heme (Fig. 1D). This result excludes the possibility of heme-mediated transcriptional effects on the per2 gene and points toward heme-mediated control of protein stability, since untreated cells showed steady levels of Per2 (Fig. 1D). To further explore the dependence of heme on Per2 stability, CHO cells were pretreated with succinylacetone, an inhibitor of δ-aminolevulinic acid dehydratase, to prevent de novo synthesis of endogenous heme (Fig. 1E). Consistent with our in vitro data, downregulation of Per2 in CHO cells was inhibited by succinylacetone but induced by further addition of exogenous hemin, indicating that heme synthesis is essential for Per2 degradation.

Heme binds within the C-terminal domain of hPer2. To identify the region on hPer2 involved in heme targeting, purified GST-hPer2 fragments [GST-hPer2(I), GST-hPer2(II), GST-hPer2(III), GST-hPer2(II-III), and GST-hPer2(V4-VII) (Fig. 2A)] were analyzed for heme-binding activity using hemin-agarose affinity chromatography (Fig. 2B). Direct interactions between heme and PAS domain-containing fragments GST-hPer2(II), GST-hPer2(III), and GST-hPer2(II-III) were detected, confirming both the role of the PAS domain in heme binding and the reliability of the method to define heme-interacting domains (Fig. 2B). Based on this result, it seems two regions within the PAS might be involved in heme binding. This can be addressed based on the functional homology among the PAS domains of the circadian NPAS2 and Per2 proteins. The PAS domain in NPAS2 typically encompasses ~150 amino acids and contains two highly degenerate 50-residue subdomains termed A and B repeats, each of which binds one molecule of heme (for a review, see reference 10). Our results show that heme is able to bind the truncated forms of PAS domain comprising either subdomain (Fig. 2 and see Fig. 4) (see Fig. S2 in the supplemental material) with equimolar stoichiometry suggesting that, like NPAS2, two independent regions within the hPer2-PAS domain are capable of heme binding. Interestingly, while the N-terminal fragment of hPer2 comprising residues 1 to 172 [GST-hPer2(I)] did not exhibit any association with heme-agarose beads (Fig. 2B, right panel), a distinct segment of the protein located within the C-terminal region, GST-hPer2(V4-VII), exhibited strong association with heme, suggesting that a heme-binding motif is located within this region.

A novel heme-regulatory motif mediates hPer2-heme interaction. Heme-protein interaction is alternatively mediated by evolutionary conserved heme-regulatory motifs where Cys-Pro residues are invariant and where there is a tendency for a hydrophobic amino acid to be in the fourth position. Inspection of the hPer2 sequence determined the presence of two putative HRMs (Fig. 3A). Interestingly, both HRMs were located within hPer2(V4-VII), a fragment that exhibits heme-binding capacity (Fig. 2). Comparative analysis of global multiple Per2 sequence alignments exhibits conserved residues clustered in the HRMs and surrounded by sequence elements of high (for SC841PA) and low (for AC962PA) conservation (Fig. 3A). Phylogenetic analyses indicate that both putative HRMs are highly conserved modules in Per2 proteins among metazoan lineages, especially in mammals, suggesting that the sequences under investigation have a comparatively young most recent common ancestor (Fig. 3A).

Unlike other heme-binding sites, HRMs establish bonding between the cysteine sulfur and the iron atom of heme (45). Accordingly, we tested whether any of the putative HRMs identified in hPer2(V4-VII) were able to directly bind heme. Untagged hPer2(V4-VII) and its SA841PA and AA962PA mutant forms were analyzed after heme addition by absorption spectroscopy (Fig. 3B). The hPer2(V4-VII) protein fragment shifted the peak of the strongest heme absorption band (388 nm), the Soret band, toward a shorter wavelength by ~19 nm
FIG. 1. Heme modulates hPer2 stability in vitro and in vivo. (A, top) 35S-labeled hPer2 ([35S]-hPer2) [35S-hPer2(II-III) in panel B and [35S-hPer2(S662A) in panel C] was added to CHO cell extracts in the absence or presence of hemin (10 μM and 100 μM) and incubated at 37°C. Aliquots were removed at 0, 1, and 2 h and resolved by SDS-PAGE and autoradiography. In other experiments, CHO extracts were preincubated with MG-132 before the addition of [35S]-hPer2 and hemin (10 μM). Bands were quantified using an AlphaImager and normalized to the input amount (at time zero in bottom panels). The figure shows data from a single experiment that was repeated three times with similar results. The arrows on the right denote radiolabeled protein. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gels.

(D) CHO cells were incubated with hemin (10 μM) for 2, 4, 6, 8, 10, or 12 h in serum-free medium. Samples were collected at the indicated times, and endogenous levels of hPer2 were analyzed by immunoblotting (top panel). Total RNA was isolated from cells harvested at each time point (in hours) and converted to cDNA in reactions that contain equivalent amounts of total RNA. Gene-specific primers (Per2 and GAPDH) were used for PCR amplification. GAPDH was used as internal control (bottom left panel).

(E) CHO cells were treated with succinylacetone (SA) to deplete cells of endogenous heme. After removal of the medium, cells were incubated with either serum-free medium (control), SA, or SA plus hemin (10 μM). Extracts were subjected to SDS-PAGE and immunoblotting. Total protein levels were monitored by either tubulin or actin expression (bottom panels).
The SA841PA mutant form of hPer2(V4-VII), but not the AA962PA mutant form, abolished the protein’s ability to shift the hemin absorption spectrum to shorter wavelengths and confirmed the essential role of Cys841 in heme binding (Fig. 3B). A second slight shift in the Soret peak (421 nm) was detected in the wild-type fragment (Fig. 3B). Whereas an additional residual shoulder was observed at a shorter wavelength for the SA841PA protein, we believe this shoulder results from excess amounts of free hemin in the sample. Our studies indicate that neither Cys841 nor Cys962 is responsible for the peak observed at 421 nm, suggesting the existence of a secondary component involved in heme binding that we later mapped between residues 1,121 and 1,255 of hPer2 (data not shown). Overall, our results pinpoint SC841PA as a novel heme-binding motif located at the C terminus of hPer2. As a direct test of the role of the SC841PA motif in heme binding, we examined whether a shorter fragment of hPer2 [GST-hPer2(V4), residues 822 to 1,255] and its HRM mutant form [GST-hPer2(V4-SA841PA)] were able to bind hemin by affinity chromatography (Fig. 3C). As expected, GST-hPer2(V4) displayed a strong interaction for hemin, and the mutation on Cys841 completely abrogated binding. Collectively, these data indicate that heme binds to hPer2 directly through Cys841.

**Binding of heme to both HRM and PAS follows a precise stoichiometry.** Further evidence of direct binding of heme to hPer2(V4) and the PAS domain-containing fragment hPer2(III) was obtained by absorption spectra and titration experiments. Among hPer2 PAS-containing fragments, hPer2(III) was chosen because of its signal intensity. Hemin binding to hPer2(V4) shifted the Soret band from 388 to 370 nm, an event that was prevented by the mutation of Cys841 to Ala, confirming Cys841 as the axial heme ligand (Fig. 4A, left panel). Since hPer2(V4) does not have any appreciable absorption between 300 and 700 nm, the observed spectral changes on free hemin are due to alterations in the electronic structure and coordination state of the heme iron caused by its interaction with hPer2(V4). To examine the specificity of heme binding, hemin was titrated with increasing amounts of GST-hPer2(V4). The absorption peak

(369 nm [Fig. 3B]), consistent with heme binding to HRMs (45). The SA841PA mutant form of hPer2(V4-VII), but not the AA962PA mutant form, abolished the protein’s ability to shift the hemin absorption spectrum to shorter wavelengths and confirmed the essential role of Cys841 in heme binding (Fig. 3B). A second slight shift in the Soret peak (421 nm) was detected in the wild-type fragment (Fig. 3B). Whereas an additional residual shoulder was observed at a shorter wavelength for the SA841PA protein, we believe this shoulder results from excess amounts of free hemin in the sample. Our studies indicate that neither Cys841 nor Cys962 is responsible for the peak observed at 421 nm, suggesting the existence of a secondary component involved in heme binding that we later mapped between residues 1,121 and 1,255 of hPer2 (data not shown). Overall, our results pinpoint SC841PA as a novel heme-binding motif located at the C terminus of hPer2. As a direct test of the role of the SC841PA motif in heme binding, we examined whether a shorter fragment of hPer2 [GST-hPer2(V4), residues 822 to 872] and its HRM mutant form [GST-hPer2(V4-SA841PA)] were able to bind hemin by affinity chromatography (Fig. 3C). As expected, GST-hPer2(V4) displayed a strong interaction for hemin, and the mutation on Cys841 completely abrogated binding. Collectively, these data indicate that heme binds to hPer2 directly through Cys841.

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of free hemin was blue shifted to 370 nm after the addition of the smallest amount of protein, whereas the amplitude of the peak increased accordingly with protein concentration (Fig. 4B, left panel). Titration curves show a well-defined inflection point corresponding to a molar stoichiometry of hemin and GST-hPer2(V4) of 1:1 (Fig. 4C). Specific binding of hemin to hPer2(V4-SA^{411}PA) was not detected by absorption experiments (Fig. 4A, left panel). Titration analysis showed initial diminution of the absorption peak of free hemin (388 nm) after hPer2(V4-SA^{411}PA) addition followed by a continuous shift around the hemin peak wavelength, likely caused by nonspecific binding by excess protein (Fig. 4B, middle panel, and C).

Evidence shows that the PAS domain in mouse Per2 (mPer2) mediates heme binding (15), but there is no spectroscopic data illustrating binding details in this domain. We determined that hPer2 PAS domain heme binding is mediated by either methionine/histidine or bis-histidine coordination, since the free hemin absorption spectra (388 nm) shifts to a Soret peak at 412 nm (Fig. 4A, right panel) (see Fig. S2 in the supplemental material). Titration experiments also defined the stoichiometry of the interaction and established that heme binds to PAS-A and -B subdomains, forming an equimolar complex in each case (Fig. 4B and C) (see Fig. S2 in the supplemental material). Altogether, these results demonstrate that (i) Fe(III)-heme binds to hPer2 at two distinct sites (SC^{841}PA motif and PAS domain), (ii) binding is mediated by different coordination in HRM and PAS, and (iii) both subdomains in PAS are able to bind heme.

Degradation of hPer2 depends exclusively on binding of oxidized heme to HRM. Because hemin interacts strongly with both PAS and HRM (Fig. 4), we next examined whether either site was able to bind the reduced form of heme. To study this possibility, hemin was reduced by the addition of sodium dithionite and incubated with GST-hPer2(III), GST-hPer2(V4), or GST-hPer2(V4-SA^{411}PA), and their interactions were monitored by absorption spectra (Fig. 5A). As with ferric heme, ferrous heme has distinct absorption characteristics that shift upon protein binding. Accordingly, a Soret peak at 421 nm was observed exclusively in the presence of GST-hPer2(III) (Fig. 5A) and GST-hPer2(II-III/PAS-B) (see Fig. S2C in the supplemental material), suggesting that only this domain is able to bind both forms of heme. Neither GST-hPer2(V4) nor GST-hPer2(V4-SA^{411}PA) exhibits any apparent peak in the spectra when incubated in the presence of a reducing agent, indicating that ferrous heme is not a suitable ligand for HRM. Therefore, we conclude that both forms of heme are able to bind PAS but that only oxidized heme binds to HRM, which suggests that this interaction takes place exclusively under specific redox conditions.

We next asked whether degradation of hPer2 depends on the redox state of the bound heme iron. To address this question, we used a cell-free system and evaluated hPer2 turnover in the presence of oxidized and reduced forms of heme (Fig. 5B). hPer2 stability was initially monitored in CHO cell extracts in the absence or presence of DTT (control). As expected, hPer2 remained stable in either condition, suggesting that factors other than heme are not required for hPer2 degradation (Fig. 5B, top panels). In agreement with Fig. 1, the sole addition of hemin (25 or 50 μM) showed an increased rate
of hPer2 degradation compared to controls (Fig. 5B, left panels). Interestingly, the stability of hPer2 was restored when hemin was preincubated with DTT before its addition to the extract, suggesting that the redox state of the heme iron is a determinant of hPer2 stability (Fig. 5B, right panels, and C). To rule out the possibility that DTT reduced proteolysis of hPer2 by a nonspecific toxic effect in the extract, we measured the degradation of a nonrelated protein (a cyclin-dependent inhibitor p27Xic1T204D) in the presence or absence of DTT (Fig. 5D). As a test of the role of HRM in hemin-mediated degradation of hPer2, we examined whether the levels of hPer2(II-III) were altered in either redox condition (Fig. 5E and F). Labeled hPer2(II-III) showed steady levels in a cell-free assay throughout the time course analyzed independently of the redox state of the heme iron (Fig. 5E and F). Thus, a heme-binding site other than PAS must be responsible for hPer2 degradation, supporting the role of HRM in mediating hPer2 stability.

It is established that reactive oxygen species encompass a variety of diverse chemical species, including superoxide anions, hydroxyl radicals, and hydrogen peroxide. These various radical species can either be generated exogenously from several different sources (i.e., radiation, hyperthermia, and growth factors) or produced intracellularly as a consequence of metabolic activities, thus perturbing the normal redox balance and shifting cells into a state of oxidative stress. Therefore, we explored the consequences of inducing oxidative stress by diverse sources from different origins on hPer2 stability. Our data show that neither the addition of hydrogen peroxide, high metal concentration, and lipopolysaccharides, nor treatment with ionizing radiation and heat shock resulted in altered levels of hPer2, suggesting that hPer2 degradation is not a general response to oxidative stress conditions (see Fig. S5 in the supplemental material). Moreover, our data point directly toward a heme-mediated response, since the addition of Fe(III), per se, did not cause hPer2 degradation in vitro, but Fe(III)-heme (hemin) addition does (see Fig. S5 in the supplemental material), suggesting that iron must be converted to heme, before it can trigger hPer2 degradation.

It has long been recognized that the conformational stability of hPer2 degradation compared to controls (Fig. 5B, left panels). Interestingly, the stability of hPer2 was restored when hemin was preincubated with DTT before its addition to the extract, suggesting that the redox state of the heme iron is a determinant of hPer2 stability (Fig. 5B, right panels, and C). To rule out the possibility that DTT reduced proteolysis of hPer2 by a nonspecific toxic effect in the extract, we measured the degradation of a nonrelated protein (a cyclin-dependent inhibitor p27Xic1T204D) in the presence or absence of DTT (Fig. 5D). As a test of the role of HRM in hemin-mediated degradation of hPer2, we examined whether the levels of hPer2(II-III) were altered in either redox condition (Fig. 5E and F). Labeled hPer2(II-III) showed steady levels in a cell-free assay throughout the time course analyzed independently of the redox state of the heme iron (Fig. 5E and F). Thus, a heme-binding site other than PAS must be responsible for hPer2 degradation, supporting the role of HRM in mediating hPer2 stability.

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It has long been recognized that the conformational stability
FIG. 5. Degradation of hPer2 depends exclusively on the redox state of the heme bound to HRM. (A) Absorption spectra of ferrous heme (inset) in the presence of GST, GST-hPer2(III), GST-hPer2(V4), and GST-hPer2(V4-SA841PA). (B) CHO cell extracts were incubated with 35S-labeled hPer2 ([35S]-hPer2) in the absence (control) or presence of hemin (25 or 50 μM). In a duplicate set of reactions, hemin was preincubated with DTT before adding radiolabeled protein. Aliquots were taken at different times and analyzed by SDS-PAGE and autoradiography. (C) Bands in panel B were quantified using an AlphaImager system, and values were normalized to the input protein (time zero). The figure shows data from a single experiment that was repeated three times with similar results. (D) 35S-labeled p27Xic1T204D ([35S]-p27Xic1T204D) was used as control of extract quality upon DTT addition. Samples were analyzed and later quantified as described above for panels B and C, respectively. (E) Cell-free assays of 35S-labeled hPer2(II-III) ([35S]-hPer2(II-III)) in the absence or presence of hemin with (+) or without (−) DTT addition were performed essentially as described above for panel B. (F) Bands in panel E were quantified as indicated above.
of a protein and its proteolytic susceptibility are linked. The reason for this linkage lies in the assumption that certain protein conformational states are better substrates for proteases, with highly ordered conformations being relatively poor substrates owing to the lack of conformational freedom of the polypeptide chain (24). Therefore, we explored whether binding of heme influences the conformation of the protein using CD spectroscopy. Experiments were carried out in the presence of increasing concentrations of hemin and the apo forms of hPer2(II-III), hPer2(V4-VII), and hPer2(V4-VII-SA841PA). Prediction of the secondary structure of hPer2(V4-VII) revealed the presence of a 24% \( \alpha \)-helical, 18% \( \beta \)-strand, and 19% \( \beta \)-turn content (Fig. 6A). The hPer2(V4-VII) secondary structure elements were disrupted after treatment of the protein with guanidium hydrochloride (Fig. 6A). hPer2(V4-VII-SA841PA) showed essentially the same overall fold as the wild-type construct, suggesting that the Cys841 mutation does not alter the overall structure of the C-terminal domain (Fig. 6B), supporting a direct role for HRM in heme binding. The N terminus of hPer2(II-III) revealed a 5% \( \alpha \)-helical, 29% \( \beta \)-strand, and 21% \( \beta \)-turn content, in agreement with the structural data from other PAS domain-containing proteins (Fig. 6C) (5). Neither hPer2(V4-VII) nor hPer2(II-III) showed a significant rearrangement of their secondary structure even at twofold excess of ligand (Fig. 6A and C), suggesting that hPer2 degradation might result from its inability to form a stable heterodimer with hCry1 rather than a ligand-induced unfolding state. To test this possibility, pull-down experiments were performed using GST-hCry1-bound beads in the presence or absence of hemin and radiolabeled hPer2 fragments (Fig. 6D). In agreement with its role in hCry1 binding, the C-terminal fragment of hPer2 showed a reduced interaction to GST-hCry1 in the presence of hemin, suggesting that competition prevents this association. The labeled PAS domain-containing protein was unable to interact with hCry1 independently of hemin addition and does restrict the heme
effect on hPer2/hCry1 formation to the C-terminal portion of hPer2.

**HRM is required for degradation of hPer2 in vivo.** To gain further insight into the role of HRM in the degradation of Per2 in vivo, we transiently transfected CHO cells with either myc-hPer2 or myc-hPer2-SA841PA and evaluated their subcellular localization and intracellular levels in response to heme addition by immunofluorescence microscopy (Fig. 7A). In agreement with previous observations (40), immunofluorescence staining showed that myc-hPer2 was distributed in both nuclear and cytosolic compartments and that its accumulation was remarkably higher in the former (Fig. 7A). The cellular distribution of myc-hPer2-SA841PA mutant was similar to that of the wild-type protein, and thus, we conclude the Cys841Ala mutation does not alter hPer2 localization (Fig. 7A). Hemin addition to myc-hPer2-transfected cells resulted in decreased levels of the nuclear protein without increasing hPer2 levels in the cytosolic compartment, suggesting that degradation, rather than translocation, was triggered by heme (Fig. 7A and B). Supporting the role of HRM in heme-mediated hPer2 degradation in vivo, the addition of heme to myc-hPer2-SA841PA-transfected cells did not result in apparent changes in mutant protein levels (Fig. 7A). Profile plotting of signal intensity along cross sections of cells transfected with wild-type and hPer2 mutants confirmed heme-induced nuclear degradation of hPer2 and unambiguously confirmed that this phenomenon is mediated by HRM (Fig. 7B).

To further support the concept that HRM is sufficient to promote heme-mediated hPer2 degradation and that heme binding to PAS domain plays a distinct role (15), we transfected cells with myc-hPer2, myc-hPer2-SA841PA, or myc-hPer2(II-III) and evaluated their total protein levels in response to heme addition (Fig. 7C). The remarkable stability of myc-hPer2(II-III) observed in the presence of heme contrasted greatly with the levels of myc-hPer2 detected under the same condition, suggesting that binding of heme to the PAS domain does not alter its stability in vivo (Fig. 7C). The presence of equivalent levels of myc-hPer2-SA841PA in the absence or presence of heme further supports our model.

**Binding of heme to HRM prevents the formation of the hPer2/hCry1 complex.** The C terminus of Per2 physically associates with Cry proteins (9, 19), and the complex translocates to the nucleus where it acts as a negative regulator by directly interacting with Clock/Bmal1 (31). Thus, we first asked whether heme treatment of cells alters the intracellular levels of the hPer2/hCry1 complex (Fig. 8). Immunoprecipitation assays of heme-treated myc-hPer2/FLAG-hCry1 cells were analyzed for the presence of heterodimers by immunoblotting (Fig. 8A). Results show reduced levels of bound hCry1 in heme-treated samples, indicating that heme alters hPer2/hCry1 levels in cells (Fig. 8A). To rule out the possibility that heme can cause hCry1 degradation and disrupt hPer2/hCry1 interaction, transfected CHO cells were incubated with heme, and hCry1 levels were monitored at different times. As shown in Fig. S3A in the supplemental material, hCry1 levels remained invariant throughout the time course analyzed, suggesting that hCry1 stability is independent of the presence of heme. A similar result was obtained when hCry1 stability was tested in the presence of heme in a cell-free assay (see Fig. S3B in the supplemental material).

We then examined whether heme binding to hPer2 prevents the formation of hPer2/hCry1 or disrupts an already preformed complex instead. To evaluate either model, we first recapitulated the cellular events leading to heme-dependent reduction of hPer2/hCry1 levels in vitro (Fig. 8B). Recombinant GST-hCry1, 35S-labeled hPer2, and heme were simultaneously incubated, and the amount of 35S-labeled hPer2 present in the complex was analyzed by pull-down experiments (Fig. 8B and 6D). As was the case with transfected cell extracts, our in vitro assay showed lower levels of 35S-labeled hPer2 associated with GST-hCry1 in the presence of heme, supporting a model where ligand binding compromises hPer2/hCry1 complex formation. Because of the nature of our in vitro assay, only two proteins and heme were present, which also suggests that heme binding to the C terminus of hPer2 prevents or disrupts its association with hCry1 and that heme-mediated degradation of hPer2 might be a consequence of lack of association.

Next, we established which event of the complex formation is inhibited by heme binding. In the first scenario, hPer2/GST-hCry1 complex was allowed to form and later incubated with heme (Fig. 8C). In a parallel experiment, hPer2 was preincubated with heme, added to GST-hCry1, and analyzed by pull-down experiments (Fig. 8C). Results demonstrate that more hPer2 is bound to GST-hCry1 when the complex is preformed, suggesting that heme is unable to disrupt a stable heterodimer.

**Expression of a non-heme-responsive HRM form of hPer2 alters the pattern of circadian gene expression.** The observation that circadian gene expression can persist for several days in serum-free medium after an initial serum shock (1, 2) prompted us to test the effects of hPer2 and hPer2(SA841PA) mutant on the mRNA accumulation profile of circadian genes. We investigated one of the known downstream effectors of Per2 signaling, Rev-erba, a transcript that is lowest at times when Per2 expression peaks in the nucleus (26). Circadian Rev-erba expression is controlled by components of the general feedback loop, thus influencing the period length and phase-shifting properties of the clock (26). In agreement with our model, cells transfected with hPer2 exhibited reduced levels of Rev-erba compared with nontransfected cells (Fig. 9A, right panel), an effect that is reversed when cells were pretreated with heme (Fig. 9A, bottom left panel). Moreover, transfection with hPer2(SA841PA) resulted in sustained downregulation of Rev-erba transcription throughout the analyzed time course (Fig. 9A, bottom right panel). As predicted, the addition of heme to hPer2(SA841PA)-transfected cells did not result in altered levels of Rev-erba, since the ligand can no longer bind the mutant protein and is therefore unable to act on its stability.

Overall, our observations favor a scenario where heme plays an essential role in controlling hPer2 cellular levels by targeting hPer2 for degradation and preventing hPer2/hCry1 complex accumulation (Fig. 9B). Our tryptophan fluorescence spectroscopy data show that both PAS and HRM bind heme with roughly equal affinity in the nanomolar range (K_d hPer2(V4-VII) [dissociation constant], 9.20 ± 0.94 nM; K_d hPer2(II-III), 12.31 ± 0.43 nM; see Fig. S4 in the supplemental material). Interestingly, whereas ferrous heme will bind only to the PAS domain (Fig. 5A), its ferric form could, in principle, target either binding site. At this point, we hypothesize that binding of ferric heme to either PAS or HRM might depend on their availability. For
FIG. 7. The HRM modulates hPer2 stability in CHO cells. (A) CHO cells were transfected with either myc-tagged hPer2 or -hPer2(SA841PA) plasmids and incubated with hemin (+). Expressed proteins and DNA were detected using a Cy3-conjugated anti-myc antibody and DAPI, respectively. (B) Profile plots of signal intensity across the cell. Recombinant protein and DNA levels were scored along the white lines shown in panel A and represented as intensity values (red for protein; black for DNA). (C) CHO cells were transfected with either myc-hPer2, -hPer2(SA841PA), or -hPer2(II-III) and treated with (+) or without (−) hemin. Cell extracts were analyzed for the presence of recombinant proteins by immunoblotting using an anti-myc antibody. Bands were quantified using an AlphaImager system, and values were normalized to β-galactosidase activity. Results similar to those presented here were observed in three independent experiments.
example, preassociation of hPer2 to hCry1 prevents the access of heme to HRM but not PAS (Fig. 8 and 9B) and thus affects signaling downstream. Accordingly, binding of heme to PAS in the mPer2/hCry1 complex regulates the transcriptional activity of Bmal1/NPAS2 and the expression of the alas1 gene (15). Conversely, the absence of hCry1 will allow heme to bind HRM (or both HRM and PAS simultaneously) and promote instability of hPer2 (this study), an event that is exclusively mediated by HRM, since heme binding to PAS does not alter hPer2 instability (Fig. 1B). In this scenario, downregulation of hPer2 directly impacts the oscillatory expression of circadian genes. Thus, this novel pathway ensures an alternative mechanism to physiologically controlling the circadian clock by acting on gene expression.

**DISCUSSION**

The mammalian circadian system influences most physiological activities, including sleep/wake cycles, cardiovascular activity, body temperature, blood pressure, glucose and fat metabolism, renal plasma flow, liver metabolism and detoxification, and hormonal secretion (32). Cross talk between the body’s circadian rhythm and metabolic systems has been identified within both the gluconeogenic and lipogenic pathways and in organisms as diverse as flies and mammals. Examples include the circadian oscillatory expression of the sterol-regulatory element-binding proteins 1a and 1c, a group of transcription factors that bind to the sterol regulatory element to control the hepatic transcriptome and thus the hepatic physiology. In addition, the orphan nuclear receptor Rev-erbα, a negative regulator of the circadian core gene bmal1, is expressed according to a robust circadian pattern and is induced during normal adipogenesis. Conversely, the retinoic acid-related orphan receptors also modulate bmal1 expression while regulating lipid flux, lipogenesis, and lipid storage in skeletal muscle, providing an additional nodal point interrelating metabolic and circadian physiology. Further studies linked carbohydrate metabolism and circadian rhythms in fruit flies, and strong evidence supports a cross talk mechanism between nuclear
FIG. 9. HRM-heme binding modulates the expression of Rev-erbs. (A) CHO cells were transfected with myc-hPer2, myc-hPer2(SA841PA), or empty vector (control) before the cells were shifted to a medium containing 50% horse serum and incubated for 2 h (time zero [see Materials and Methods]). Synchronized cells were then maintained in 5 mM succinylacetone followed by hemin (10 μM) addition (at 24 h). Total RNA was prepared from about 10^7 cells at the times (in hours after serum shock) indicated in the panels, and the relative levels of Rev-erb and GAPDH were determined by reverse transcription-PCR. Rev-erb levels were normalized to those of the housekeeping gene. (B) A proposed model for the role of heme binding in hPer2/hCry1 complex formation is depicted. C, C terminus; N, N terminus, ub, ubiquitin; Fe^{3+}, ferric heme; Fe^{2+}, ferrous heme.
hormone receptors and the core circadian complex Clock/Bmal1 in adipogenesis (for a review, see reference 18).

An additional level of complexity arises from experiments showing that many heme-containing molecules regulate cellular homeostasis which, consistent with the circadian oscillatory nature of heme levels, led us to propose heme as a candidate bridge molecule for the circadian and metabolic mechanisms. We and other groups have reported that heme directly targets circadian clock components modulating both gene transcription and protein stability (6, 15, 43; this article). We established that heme directly binds to a novel regulatory motif in hPer2 in a redox-dependent manner, resulting in hPer2 instability and altered hPer2/hCry1 formation. Therefore, we propose that hPer2 acts as a heme sensor-transducer molecule, coupling metabolic signals to the circadian oscillator.

Control of Per2 stability plays a key role in driving circadian rhythmicity (31). During the transcription-translation-feedback loop, Per2 is rapidly degraded as a result of phosphorylation by the double-time kinase in Drosophila (27) or CKIε in mammals (3), altering the levels of Per2 available for heterodimerization and nuclear translocation. Although phosphorylation remains the primary mechanism responsible for Per2 degradation, alternative mechanisms to control its stability might exist. We tested the simplest model in which binding of heme to hPer2 induces protein instability in a phosphorylation-independent fashion. Indeed, heme favors hPer2 degradation both in vitro and in vivo. More importantly, this event is independent of both phosphorylation by CKIε and binding of heme to the PAS domain, indicating that degradation of hPer2 can occur by alternative mechanisms. Period protein turnover is mediated by ubiquitination and further degradation by the proteasome pathway (39). Our data agreed, showing that inhibitors of proteasome function restore hPer2 levels, supporting a model where heme-mediated degradation of hPer2 depends on ubiquitination. Similarly, heme-mediated ubiquitination and degradation exist in iron regulatory proteins in other systems (13). Specifically, IRP2 oxidation, which is mediated by heme binding to its regulatory domain, triggers IRP2 ubiquitination-dependent degradation regulating the expression of genes involved in iron metabolism (13, 14, 41). In addition, the DNA-binding activity of the transcriptional repressor Bach1 dramatically decreases upon heme binding through multiple HRMs (23, 36), inducing nuclear export of Bach1 (37), polyubiquitination, and degradation of the repressor (44). Heme also binds to the bacterial iron response regulator through two distinct regions including an HRM, a necessary interaction for normal degradation (28, 29, 42). In this scenario, both redox states are required for rapid turnover of Irr, although its stability is independent of ubiquitination and likely mediated by an unknown specific protease (42). Like IRP2 and Irr, heme-dependent degradation of hPer2 is mediated by a CP core of a HRM. Unlike IRP2, where the Cys and His residues within the HRM participate in coordination and are responsible for axial ligand of ferric and ferrous heme (13), the HRM of hPer2 lacks the His component found in the HRM of IRP2 and exclusively binds ferric heme. More importantly, whereas oxidized heme binds to both HRM and PAS of hPer2, it is only its interaction with the former that is responsible for hPer2 degradation. This is the first demonstration of ligand-induced instability of a clock gene product and is a novel mode of regulation of the circadian feedback loop.

To understand the mechanism underlying heme-hPer2 recognition, we studied whether conformational changes are associated with ligand binding and heterodimeric complex formation. It is not known whether or to what extent heme binding to hPer2 plays a role in hPer2/hCry1 complex formation. Examples show slight secondary structural changes in helicity in the electron transport protein cytochrome b$_{562}$ upon heme binding (16), whereas large changes in secondary structure are revealed when the His-rich protein II is compared to the apoprotein after ferric heme addition (33). Our secondary structural studies of the C-terminal domain of hPer2 show that heme binding does not induce major conformational changes in the protein, suggesting that degradation of hPer2 does not result from unfolding upon ligand binding but is most likely mediated by an unknown, specific ubiquitin ligase enzyme. Much has been done to identify the molecules responsible for selective recognition of oxidized target proteins, including the recent characterization of the heme-oxidized IRP2 ubiquitin ligase-1 responsible for IRP2 turnover (13, 41). Interestingly, mPer2 ubiquitination is reduced by its interaction with Cry and is mediated by the Cry-binding domain residing in the C-terminal portion of mPer2 (9, 19), a mode of regulation closely resembling the organization of the Per/Tim loop in Drosophila (17).

All of these findings raise the question of whether heme binding to the C terminus of hPer2 prevents the formation of the hPer2/hCry1 complex or rather perturbs the stability of an already preformed heterodimer. Here, we provide evidence that heme acts by preventing hPer2 from binding to hCry1 when bound to HRM, whereas heme-PAS binding neither promotes hPer2 degradation nor affects hCry1 association. Heme binding to PAS plays a role in mPer2 interaction with the Bmal1/NPAS2 complex and in its transcriptional activity (15). Accordingly, cyanocobalamin, a vitamin B$_{12}$ analogue with a similar porphyrin ring structure to heme, greatly decreases the binding of NPAS2 and mPer2 to a heme-agarose matrix (15). The overall data are reconciled in a model where heme binding to either HRM or PAS in hPer2 targets different circadian complexes for regulation, likely connecting the cellular response to changes in heme levels. Furthermore, we propose that selectivity of binding is dictated by the redox state of the iron core in the heme molecule. Last, we demonstrate that transcription of the orphan nuclear receptor Rev-erba, a major regulator of the circadian oscillator that influences period length and affects the phase-shifting properties of the clock, is responsive to heme binding to the HRM of hPer2. These experiments add a new level of regulation in circadian gene expression by directly coupling metabolic sensing to the transcriptional control of the molecular oscillator.

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J.Y. performed all experiments except those mentioned below. A.L. and D.G.S.C. performed and analyzed CD experiments. C.V.F. performed immunofluorescence experiments, and K.D.K. performed the in vitro hPer2/hCRY1 binding and fluorescence spectroscopy experiments. D.G.S.C. processed and analyzed the fluorescence data. K.E.D., C.S.S., and S.P.M. performed the experiments described in the supplemental material and provided technical support at various stages of the project. C.V.F., D.G.S.C., and J.Y. analyzed the overall data. C.V.F. wrote the manuscript.

REFERENCES

Supplemental Material

Materials and Methods.

Tryptophan fluorescence analysis. Fluorescence spectra were obtained with a Jasco J-815 fluorometer at 23ºC. All spectra were recorded in a 3 ml quartz cuvette in 50 mM Tris-HCl pH 8.0, 250 mM NaCl, 1 mM EDTA. The final concentrations of the protein samples were 0.125 μM. The excitation wavelength was set to 295 nm, and emission scans were recorded in the range of 310-410 nm. Analysis of the apparent dissociation constants (K_d) were performed by nonlinear regression and the hemin binding titration data were fitted to the equation below:

\[ F = \frac{F_{max} \cdot L}{K_d} + L \]

where \( F \) is the observed fluorescence, \( F_{max} \) is the maximum amplitude of fluorescence quenching, \( L \) is the total heme concentration, and \( K_d \) is the apparent dissociation constant. Calculations were performed using the Microsoft Excel Solver software.

In vitro oxidative stress assay. For in vitro degradation of hPer2 in response to oxidative stress conditions, \([^{35}S]-hPer2\) was incubated with CHO cell extracts prepared in lysis buffer supplemented with an energy regenerating system as described in the Materials and Methods section. Extracts were incubated with hemin (10 μM and 100 μM), FeCl_3 (10 μM and 100 μM), H_2O_2 (0.1 mM and 1 mM) or lipopolysaccharides (1 ng/ml) at 37°C and aliquots removed at the indicated times. In other stress assays, CHO cells were subjected to 5 Gy of ionizing radiation (Co^{60} source) or heat shock (10 min at 42°C) before the extract was prepared. Samples were taking at different times during incubation and reactions were stopped by addition of Laemmli sample buffer, resolved by SDS-PAGE and visualized by autoradiography.

Reverse transcription. One microgram of total RNA was added to a reaction containing 50 mM Tris–HCl pH 8.3, 10 mM DTT, 0.0225 OD_{260} units of random hexamers, 3.5 μg of bovine serum albumin, 3 mM MgCl_2, 0.5 mM of the deoxynucleotide triphosphates, 30 units of RNAguard RNase inhibitor, 500 units of modified MMLV reverse transcriptase (BioRad) in 20 μl final volume. Reactions were incubated at 25°C for 5 min, then at 42°C for 30 min followed by 85°C for 5 min, to inactivate the reverse transcriptase.
Figure Legends.

Supplementary Figure 1. Mdm2 levels remain stable in the presence of heme. [35S]-Mdm2 was added to CHO cell extracts in the absence or presence of hemin (10 μM and 100 μM) and incubated at 37°C. Aliquots were removed at 0, 1 and 2h and resolved by SDS-PAGE and autoradiography. The figure shows data from a single experiment that was repeated twice with similar results. Arrow on the right denote radiolabeled protein. Molecular mass markers (in kDa) are indicated on the left.

Supplementary Figure 2. Heme binds to PAS-A subdomain of hPer2 with equimolar stoichiometry A. Absorption spectra of heme after addition of increasing concentration of GST-hPer2(II-IIIΔPAS-B) (residues 173-574Δ322-374) up to 8 mol equivalent of the heme amount (black arrow). Free heme spectrum is indicated with a red arrow. B. Titration curve of heme with increasing amounts of the indicated protein is represented as absorbance at 417 nm (GST-hPer2(IIIΔPAS-B)) as a function of the molar ratios of the protein to heme. C. Absorption spectra of ferrous heme in the presence of GST-hPer2(II-IIIΔPAS-B).

Supplementary Figure 3. hCry1 levels remain stable in the presence of heme. A. CHO cells were transfected with pCS2+myc-hCry1 and incubated with succinylacetone (5 mM) for 24h before hemin (10 μM) addition as indicated in the Materials and Methods section. Cells were collected at indicated times and extracts were lysed and analyzed for hCry1 levels by SDS-PAGE and immunoblotting (upper panel). Total protein levels were monitored by tubulin expression (lower panels). B. [35S]-myc-hCry1 was added to CHO cell extracts in the absence or presence of heme (10 μM and 100 μM) and incubated at 37°C for the indicated times. Aliquots were removed and resolved by SDS-PAGE and autoradiography.

Supplementary Figure 4. Tryptophan fluorescence studies of heme-hPer2 interaction. Tryptophan fluorescence of hPer2(V4-VII) ( ) and hPer2(II-III) (Δ) was measured at 338 and 335 nm, respectively in the absence and presence of different heme concentrations (1 nM-75 nM). Hemin-mediated protein quenching was plotted as 100-[(F/Fo)x100], where F was the observed fluorescence intensity at a certain heme concentration and Fo was the initial fluorescence intensity in the absence of heme. The apparent Kd values were calculated from the fitting of the data to a nonlinear regression equation as described in Supplementary Material and Methods section.

Supplementary Figure 5. hPer2 stability in response to oxidative stress conditions. Radiolabeled hPer2 was added to CHO cell extracts in the absence or presence of hemin (Fe(III)-heme; 10 μM and 100 μM) and various concentrations of oxidative stress-inducer agents such as FeCl3 (Fe(III); 10 and 100 μM), H2O2 (0.1 and 1 mM) and lipopolysaccharides (LPS, 1 ng/ml). In other experiments, CHO cells were treated with 5 Gy of ionizing radiation or preincubated at 42°C for 10 min before harvesting. Extracts were then prepared as described in the Materials and Methods section. Aliquots were taken at the indicated time and reactions stopped by the addition of Laemmli sample buffer. Samples were resolved by SDS-PAGE and autoradiography. The arrows on the right denote radiolabeled hPer2.
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<th>H₂O₂ (mM)</th>
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C. **Phosphorylation in the PCNA domain of p27Xic1 is required for cell cycle progression in early Xenopus development.** Rebecca Sinnott, Sean P. Mury, Andrew Lucas, Karen E. Drahos, Daniel G. S. Capelluto, Carla V. Finkielstein. *In preparation.*

In contribution to this project, I have performed microinjections, fixed, and photographed embryos in order to define the effect of specific point mutations in p27Xic1 *in vivo.*

**Figure 1** Sinnott *et al.:* Mutation of Thr204 to Ala enhances p27Xic1-mediated cell cycle arrest, but mutation to Asp attenuates arrest. Fertilized *Xenopus laevis* embryos were microinjected with 5 ng of p27Xic1 protein constructs at St.1. Embryos were fixed when controls reached St.5.

**Figure 2** Sinnott *et al.:* Mutation of Ser92 abrogates p27Xic1-mediated cell cycle arrest, but mutation of Thr172 increases arrest. Fertilized *Xenopus laevis* embryos were microinjected at St.1 with 5 ng of the indicated proteins. Embryos were fixed when controls reached St.5.
Appendix A: IRB Approval CF-08-196-IR

- April 2, 2008 – April 1, 2009
- April 2, 2009 – April 1, 2010
DATE: April 2, 2008

MEMORANDUM

TO: Carla Finkielstein
Karen Drahos
John Welsh

FROM: David M. Moore

SUBJECT: IRB Expedited Approval: “A Novel Ligand Drives Lipid Competition for the Disabled-2 PID Domain”, IRB # 08-196

This memo is regarding the above-mentioned protocol. The proposed research is eligible for expedited review according to the specifications authorized by 45 CFR 46.110 and 21 CFR 56.110. As Chair of the Virginia Tech Institutional Review Board, I have granted approval to the study for a period of 12 months, effective April 2, 2008.

As an investigator of human subjects, your responsibilities include the following:

1. Report promptly proposed changes in previously approved human subject research activities to the IRB, including changes to your study forms, procedures and investigators, regardless of how minor. The proposed changes must not be initiated without IRB review and approval, except where necessary to eliminate apparent immediate hazards to the subjects.

2. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

3. Report promptly to the IRB of the study’s closing (i.e., data collecting and data analysis complete at Virginia Tech). If the study is to continue past the expiration date (listed above), investigators must submit a request for continuing review prior to the continuing review due date (listed above). It is the researcher’s responsibility to obtain re-approval from the IRB before the study’s expiration date.

4. If re-approval is not obtained (unless the study has been reported to the IRB as closed) prior to the expiration date, all activities involving human subjects and data analysis must cease immediately, except where necessary to eliminate apparent immediate hazards to the subjects.

Important:
If you are conducting federally funded non-exempt research, please send the applicable OSP/grant proposal to the IRB office, once available. OSP funds may not be released until the IRB has compared and found consistent the proposal and related IRB applicaton.

cc: File
DATE: March 11, 2009

MEMORANDUM

TO: Carla Finkielstein
    Karen Drahos
    John Welsh

FROM: David M. Moore

SUBJECT: IRB Expedited Continuation 1: "A Novel Ligand Drives Lipid Competition for the Disabled-2 PID Domain", IRB # 08-196

This memo is regarding the above referenced protocol which was previously granted expedited approval by the IRB. The proposed research is eligible for expedited review according to the specifications authorized by 45 CFR 46.110 and 21 CFR 56.110. Pursuant to your request, as Chair of the Virginia Tech Institutional Review Board, I have granted approval for extension of the study for a period of 12 months, effective as of April 2, 2009.

Approval of your research by the IRB provides the appropriate review as required by federal and state laws regarding human subject research. As an investigator of human subjects, your responsibilities include the following:

1. Report promptly proposed changes in previously approved human subject research activities to the IRB, including changes to your study forms, procedures and investigators, regardless of how minor. The proposed changes must not be initiated without IRB review and approval, except where necessary to eliminate apparent immediate hazards to the subjects.
2. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.
3. Report promptly to the IRB of the study’s closing (i.e., data collecting and data analysis complete at Virginia Tech). If the study is to continue past the expiration date (listed above), investigators must submit a request for continuing review prior to the continuing review due date (listed above). It is the researcher’s responsibility to obtain re-approval from the IRB before the study’s expiration date. If re-approval is not obtained (unless the study has been reported to the IRB as closed) prior to the expiration date, all activities involving human subjects and data analysis must cease immediately, except where necessary to eliminate apparent immediate hazards to the subjects.

Approval date: 4/2/2009
Continuing Review Due Date: 3/18/2010
Expiration Date: 4/1/2010

cc: File
Appendix B: Literature Cited


