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Role and Regulation of the Actin-Regulatory Protein Hs1 in Tcr Signaling

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Abstract
Numerous aspects of T cell function, including TCR signaling, migration, and execution of effector functions, depend on the actin cytoskeleton. Cytoskeletal rearrangements are driven by the action of actin-regulatory proteins, which promote or antagonize the assembly of actin filaments in response to external cues. In this work, we have examined the regulation and function of HS1, a poorly-understood actin regulatory protein, in T cells. This protein, which becomes tyrosine phosphorylated upon T cell activation, is thought to function primarily by stabilizing existing branched actin filaments. Loss of HS1 results in unstable actin responses upon TCR engagement and defective Ca2+ responses, leading to poor activation of the IL2 promoter. TCR engagement leads to phosphorylation of HS1 at Tyr 378 and Tyr 397, creating binding sites for SH2 domain-containing proteins, including Vav1 and Itk. Phosphorylation at these residues is required for Itk-dependent recruitment of HS1 to the IS, Vav1 IS localization, and HS1-dependent actin reorganization and IL2 production. Therefore, in addition to directly interacting with branched actin filaments, HS1 regulation of Vav1 localization provides another mechanism by which HS1 signals to the actin cytoskeleton. Analysis of Ca2+ responses in HS1-/- T cells reveals that the defect in these cells lies at the level of release from intracellular stores. Phosphorylated HS1 interacts with PLCγ1, and mediates its association with the cytoskeleton and regulates microcluster dynamics. Although HS1 is clearly important for signaling downstream of the TCR, HS1-/- mice exhibit normal T cell development and normal peripheral populations. Surprisingly, HS1 is not required for T cell homing to lymphoid organs, for TCR endocytosis or for CD8+ T cell effector function. However, T cells from HS1-/- mice produce reduced amounts of Ifnγ, and are subsequently less likely to become Ifnγ-producing TH1 effector cells. These data demonstrate that HS1 functions as a cytoskeletal adaptor protein and plays specific roles downstream of TCR engagement.

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Abstract

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Esteban Carrizosa

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Numerous aspects of T cell function, including TCR signaling, migration, and execution of effector functions, depend on the actin cytoskeleton. Cytoskeletal rearrangements are driven by the action of actin-regulatory proteins, which promote or antagonize the assembly of actin filaments in response to external cues. In this work, we have examined the regulation and function of HS1, a poorly-understood actin regulatory protein, in T cells. This protein, which becomes tyrosine phosphorylated upon T cell activation, is thought to function primarily by stabilizing existing branched actin filaments. Loss of HS1 results in unstable actin responses upon TCR engagement and defective Ca^{2+} responses, leading to poor activation of the *IL2* promoter. TCR engagement leads to phosphorylation of HS1 at Tyr 378 and Tyr 397, creating binding sites for SH2 domain-containing proteins, including Vav1 and Itk. Phosphorylation at these residues is required for Itk-dependent recruitment of HS1 to the IS, Vav1 IS localization, and HS1-dependent actin reorganization and IL2 production. Therefore, in addition to directly interacting with branched actin filaments, HS1 regulation of Vav1 localization provides another mechanism by which HS1 signals to the actin cytoskeleton. Analysis of Ca^{2+} responses in $HS1^{-/-}$ T cells reveals that the defect in these cells lies at the level of release from intracellular stores. Phosphorylated HS1 interacts with PLCγ1, and mediates its

association with the cytoskeleton and regulates microcluster dynamics. Although HS1 is clearly important for signaling downstream of the TCR, $HS1^{-/-}$ mice exhibit normal T cell development and normal peripheral populations. Surprisingly, HS1 is not required for T cell homing to lymphoid organs, for TCR endocytosis or for $CD8^{+}$ T cell effector function. However, T cells from $HS1^{-/-}$ mice produce reduced amounts of Ifnγ, and are subsequently less likely to become Ifnγ-producing T_H1 effector cells. These data demonstrate that HS1 functions as a cytoskeletal adaptor protein and plays specific roles downstream of TCR engagement.

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List of abbreviations

Abbreviation	Full Name
AP-1	Activator protein 1
APC	Antigen Presenting Cell
ATP	Adenosine triphosphate
BCR	B cell antigen receptor
Ca^{2+}	calcium
CRAC	Calcium release-activated calcium
Csk	C-src tyrosine kinase
CTL	Cytotoxic T Lymphocyte
CytoD	Cytochalasin D
DAG	Diacylglycerol
ER	Endoplasmic reticulum
FcεRI	Fcε Receptor I
FcγRIIIA	Fcγ receptor III A
FRET	Förster resonance energy transfer
GADS	Grb2-related adaptor downstream of Shc
Grb2	Growth factor receptor-bound protein 2
HS1	Hematopoeitic cell-specific lyn substrate 1
HTH	Helix-turn-helix
Ifnγ	Interferon gamma
IL2	Interleukin 2
IL4	Interleukin 4
$Ins(1,4,5)P_3$	Inositol (1,4,5) trisphosphate
IS	Immunological synapse
Itk	IL2-inducible T cell kinase
LAT	Linker for activation of T cells
Lck	Leukocyte-specific protein tyrosine kinase
MHC	Major histocompatibility complex
MTOC	Microtubule organizing center
Nck	Non-catalytic region of tyrosine kinase
NF-AT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa B
NTA	N-terminal acidic region
PI3K	Phosphoinositide 3-kinase
PLCγ1	Phospholipase C-gamma 1
PMA	Phorbol 12-myristate 13-acetate
PRR	Proline-rich region
$PtIns(3,4,5)P_3$	Phosphatidylinositol 3,4,5 Trisphosphate

PtIns(4,5)P_2	Phosphatidylinositol 4,5 Bisphosphate
SH2	Src homology 2
SH3	Src homology 3
SLE	Systemic Lupus Erythematosus
Slp76	SH2-domain containing leukocyte protein of 76 kDa
TCR	T cell antigen receptor
WASp	Wiskott aldrich syndrome protein
WAVE2	WASp family verprolin homologous protein 2
WT	wild-type
Zap70	Zeta chain-associated protein - 70 kDa

List of electronic files

File	Format	Size
Supplemental movie 2.1	Quicktime .mov file	532 KB
Supplemental movie 2.2	Quicktime .mov file	872 KB
Supplemental movie 2.3	Quicktime .mov file	1.5 MB
Supplemental movie 2.4	Quicktime .mov file	1.2 MB
Supplemental movie 2.5	Quicktime .mov file	804 KB
Supplemental movie 2.6	Quicktime .mov file	654 KB
Supplemental movie 2.7	Quicktime .mov file	1.3 MB
Supplemental movie 2.8	Quicktime .mov file	116 KB
Supplemental movie 2.9	Quicktime .mov file	476 KB
Supplemental movie 3.1	Quicktime .mov file	682 KB
Supplemental movie 3.2	Quicktime .mov file	2 MB
Supplemental movie 3.3	Quicktime .mov file	1.2 MB
Supplemental movie 3.4	Quicktime .mov file	1 MB
Supplemental movie 3.5	Quicktime .mov file	1.2 MB
Supplemental movie 3.6	Quicktime .mov file	692 KB
Supplemental movie 3.7	Quicktime .mov file	3.2 MB
Supplemental movie 3.8	Quicktime .mov file	454 KB
Supplemental movie 3.9	Quicktime .mov file	512 KB
Supplemental movie 3.10	Quicktime .mov file	1.9 MB

Chapter 1: Introduction[1]

Regulated reorganization of the actin cytoskeleton is important for multiple aspects of T cell function, including signaling and differentiation along discrete developmental lineages, migration through tissues, and execution of effector functions. Early studies testing the role of actin in T cell activation were based on the use of actin-disrupting agents such as cytochalasin D (CytoD). These studies showed that actin is required for adherence to target cells and cytotoxic activity (Henney and Bubbers, 1973), as well as for signaling events associated with Ca^{2+} flux and downstream changes in gene expression (Valitutti et al., 1995). However, these studies revealed a complex role for actin. For example, in T cells stimulated with superantigen-pulsed antigen presenting cells (APCs), CytoD-treatment results in diminished Ca^{2+} flux, but stimulation with anti-CD3 antibodies results in a prolonged Ca^{2+} response (Valitutti et al., 1995). Similarly, while one study showed that CytoD treatment impairs the activation of the IL2 promoter (Holsinger et al., 1998), another found that at low doses of CytoD, Ca^{2+} signaling is prolonged and IL2 production increases (Rivas et al., 2004). These findings and more recent data described below suggest that actin filaments play a dual role, enhancing T cell activation by promoting conjugate formation and the assembly of signaling complexes,

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[1] This chapter is a modified version of a review article titled "The Actin Cytoskeleton in T Cell Activation." It was published in the 2008 issue of Annual Review of Immunology, Volume 26, pages 233-259, with the following author list: Janis K. Burkhardt, Esteban Carrizosa, and Meredith H. Shaffer.

but also down-regulating activation, perhaps by facilitating molecular movements that culminate in the internalization of the TCR.

Interaction with APCs and immunological synapse formation

Upon interaction with an APC bearing appropriate MHC-peptide complexes, the T cell extends large pseudopodia and lamellipodia toward the APC, a process resulting from the recruitment and localized activation of actin polymerizing and actin binding proteins. This process results in the formation of a flattened, F-actin rich interface with the APC. In parallel with these shape changes, the MTOC and associated secretory organelles reorient within the T cell cytoplasm, and come to lie just beneath the plasma membrane, near the center of the APC contact site. This reorientation of T cell cytoskeletal elements was observed by several investigators in the early 1980s, and was recognized as a hallmark of productive T cell activation and a prerequisite for directed cytolysis and T cell help (Kupfer et al., 1985; Kupfer et al., 1994; Ryser et al., 1982).

Actin responses at the T cell-APC contact site are important for organization of signaling molecules at this site, to form the immunological synapse (IS) (Campi et al., 2005; Sims et al., 2007; Tskvitaria-Fuller et al., 2003; Wulfing et al., 2000). Protein segregation within the IS was initially described as forming a "supramolecular activation cluster (SMAC)" comprised of a central region (c-SMAC) containing TCR and associated signaling proteins within a peripheral ring (p-SMAC) containing LFA-1 and associated adhesion molecules (Monks et al., 1998). Recent studies have shown, however, that IS architecture and dynamics vary greatly depending on several variables,

including the nature of the T cell and the APC, agonist strength, costimulatory interactions, and the tissue context in which the interaction is taking place (Friedl et al., 2005; Jacobelli et al., 2004). Our understanding of IS function has evolved considerably over the past decade. Once thought to facilitate TCR signaling by bringing interacting molecules together, the c-SMAC was subsequently shown to be a site where signaling is terminated by internalization of TCR signaling complexes (Cemerski et al., 2007; Mossman et al., 2005; Varma et al., 2006). The role for the c-SMAC in downregulating T cell signaling is supported by studies in which signaling is enhanced under conditions in which c-SMAC formation is blocked and inhibited under conditions in which c-SMAC formation is induced (Cemerski et al., 2007; Lee et al., 2003; Mossman et al., 2005). An interesting study combining empirical analysis with mathematical modeling has shown that both ideas are probably correct, and that parameters such as peptide-MHC half life determine the balance between these two interrelated facets of IS function (Cemerski et al., 2007). A more recent study supports and refines this idea, suggesting that c-SMAC formation serves to regulate signal strength (Cemerski et al., 2008). Enforced c-SMAC formation reduces proliferative responses for strong agonists, but enhances responses for weak agonists. Moreover, stimulation with low doses of antigenic peptide results in detectable signaling in the c-SMAC, whereas with high peptide doses, signaling is detectable only in the p-SMAC.

Video analysis of molecular movements during IS formation show that the TCR and associated signaling molecules form microclusters around the periphery of the cell-cell contact site, and converge to form the c-SMAC (Bunnell et al., 2002; Campi et al.,

2005; Varma et al., 2006; Yokosuka et al., 2005). Sustained signaling occurs in the peripheral clusters, while central clusters are destined for degradation, at least in the context of a strong signal. Microcluster formation and centripetal movement are actin-dependent processes, controlled in part by Cdc42 (Campi et al., 2005; Tskvitaria-Fuller et al., 2003; Varma et al., 2006). Once formed, however, these signaling complexes are highly stable and are largely unaffected by actin-depolymerizing agents. It will be interesting to learn how specific actin regulatory molecules work to orchestrate microcluster formation and movement, especially as this relates to parameters such as peptide-MHC half life and T cell subset.

CONTROL OF ACTIN DYNAMICS AT THE IMMUNOLOGICAL SYNAPSE

Engagement of the TCR activates multiple actin regulatory proteins that work in concert to drive actin polymerization at the IS (Figure 1.1). The best understood of these molecules function downstream of Vav1 and other GEFs, which activate the Rho GTPases Rac1 and Cdc42 at sites of TCR engagement.

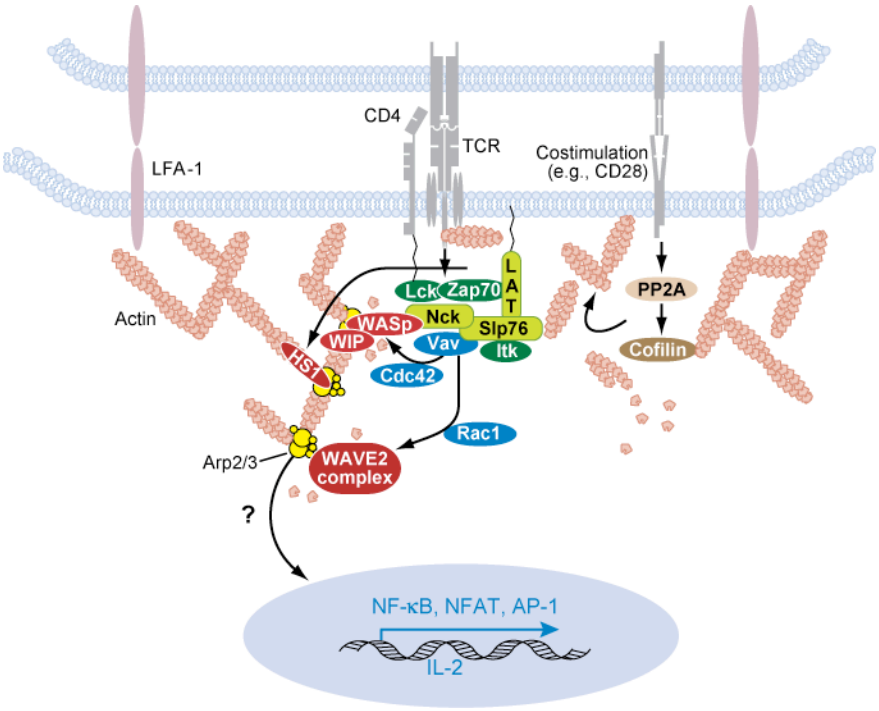

Figure 1.1: TCR signaling pathways leading to actin polymerization

Encounter of an APC induces a signaling cascade comprised of tyrosine kinases (dark green), adaptor proteins (yellow), and immediate upstream actin regulatory proteins (dark blue), that transduce signals to several nucleation promoting factors (red) that direct the polymerization of branched actin filaments at sites of TCR engagement. Engagement of costimulatory molecules leads to activation of proteins that sever actin filaments (peach), creating new barbed ends as substrates for filament growth. Question marks indicate the unknown mechanisms by which actin remodeling leads to IL2 promoter activation and other changes in gene expression.

Arp2/3 complex-dependent actin regulatory proteins

The initiation of actin filament formation is a kinetically unfavorable process, requiring the action of nucleation-promoting factors (NPFs) to generate a "seed" from which a filament can elongate. Many of the best-understood NPFs work in concert with the 7-subunit Arp2/3 complex to form new actin branches on the sides of existing actin filaments (Goley and Welch, 2006) (Figure 1.2). This complex, which is composed of two actin-related proteins, Arp2 and Arp3, and five other proteins, ARPC1-5, binds to the side of an existing actin filament, where Arp2 and Arp3 mimic two actin monomers as part of the core nucleus. NPFs such as WASp and WAVE2 then bind to Arp2/3 complex and present an actin monomer, allowing elongation to take place. Using an RNAi-based strategy, the Billadeau group showed that suppression of either Arp2 or Arp3 in Jurkat T cells results in degradation of other complex components (Gomez et al., 2007). Arp2/3 complex-deficient cells do not polymerize branched actin at the IS, and they fail to form lamellipodia in spreading assays using anti-TCR coated coverslips. Interestingly, these cells generate highly dynamic F-actin-rich filopodia in response to either APCs or anti-TCR coated surfaces, a finding that points toward additional, Arp2/3 complex-independent actin regulatory pathways. Nonetheless, this study demonstrates that Arp2/3 complex and its upstream activators are central effectors of actin polymerization at the IS.

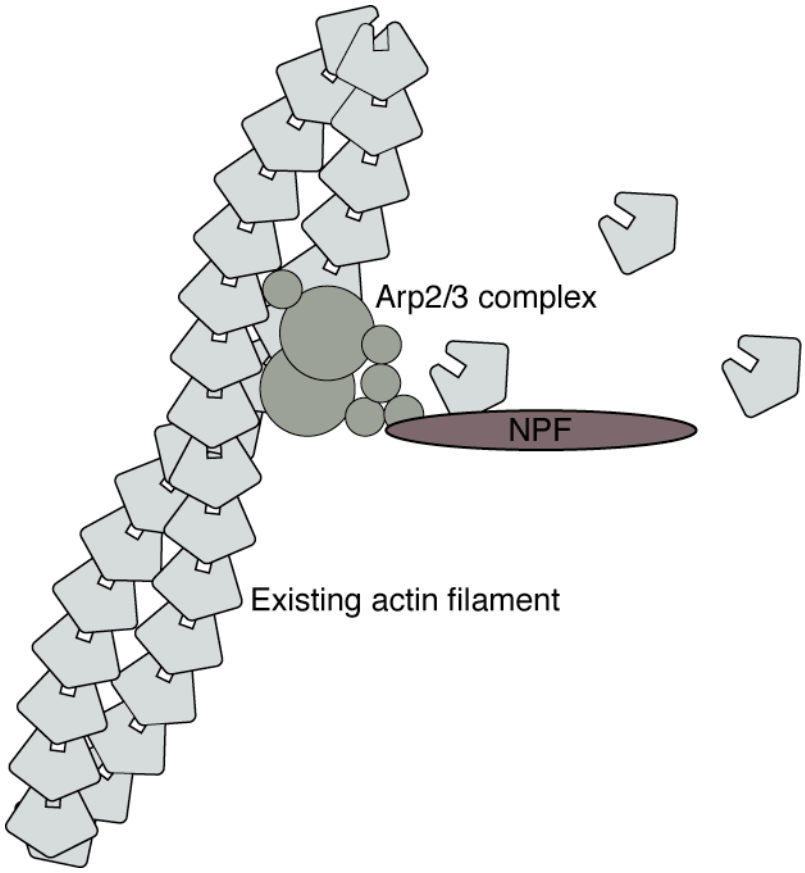

Figure 1.2: Model for branched actin polymerization by Arp2/3 complex.

Model for branched actin polymerization by Arp2/3 complex. Arp2/3 complex complex nucleates a new actin filament at a 70º angle from the side of an existing filament. NPFs function by binding to and inducing a conformational change in Arp2/3 complex and presenting actin monomers to form the new filament (Goley and Welch, 2006).

WASp

The best-characterized activator of Arp2/3 complex is WASp, named for its causative role in Wiskott-Aldrich syndrome (WAS), a severe X-linked immune deficiency disorder (Derry et al., 1994; Orange et al., 2004). The characteristic symptoms are thrombocytopenia, eczema, and recurrent infections. Patients are also prone to malignancy and autoimmune disease. Recent studies have shown that WASp is required for the function and, in certain contexts, the development of regulatory T cells (Adriani et al., 2007; Humblet-Baron et al., 2007; Maillard et al., 2007; Marangoni et al., 2007).

The domain structure of WASp is shown in Figure 1.3. The N-terminal WASp-homology region (WH1) binds constitutively to WASp interacting protein (WIP). Disruption of this interaction results in degradation of WASp (de la Fuente et al., 2007; Volkman et al., 2002). Most mutations associated with WAS are found in this region and interfere with WIP binding (Notarangelo and Ochs, 2003). WIP is almost certainly an important actin regulatory protein in its own right, but WIP function per se has not been tested, presumably because of the challenges associated with constructing WIP-deficient cells that express normal levels of WASp. However, evidence that WIP and WASp play non-redundant roles comes from analysis of WIP/WASp double-knockout mice: T cells lacking both proteins migrate less well than T cells from either of the single knockouts (Gallego et al., 2006). WASp recruitment and activation at the IS are relatively well understood.

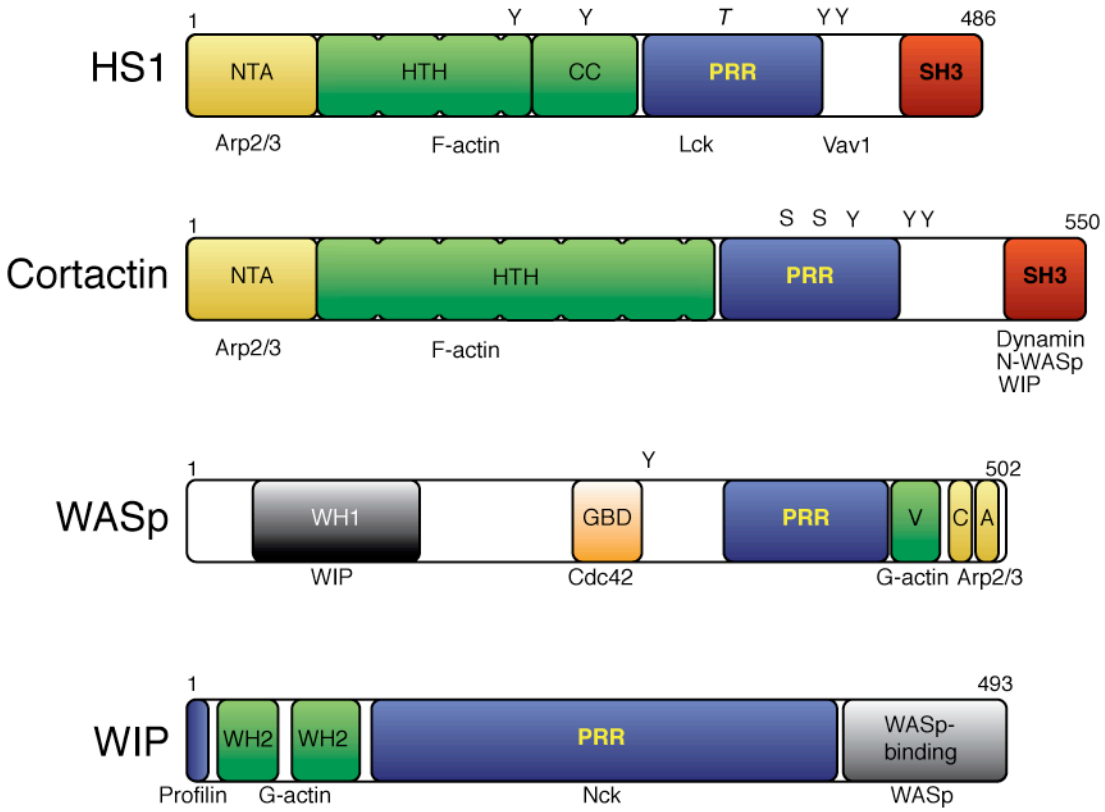

Figure 1.3: Domain structures of selected Arp2/3 complex regulators.

The domain structure of key Arp2/3 activating molecules is shown, with related regions colored similarly. Important binding partners are listed below the domains to which they bind. Known tyrosine (Y) and serine (S) phosphorylation sites are indicated, a predicted threonine phosphorylation site is indicated in italics (*T*), and total amino acid numbers are shown at the top right of each.

Abbreviations: NTA – N-terminal acidic region; HTH – Helix-turn-helix; PRD – Proline rich domain; SH3 – Src homology 3; WH1 – Wasp homology 1; GBD – GTPase binding domain; VCA – verprolin, cofilin homology, acidic region; WHD – WAVE homology domain; WH2 – WASP homology 2

Recruitment occurs through an interaction between the WASp proline-rich domain (PRD) and the SH3 domains of the adaptors Nck and PST-PIP (Badour et al., 2003; Zeng et al., 2003). Localization to the IS facilitates WASp contact with Cdc42-GTP, which binds to the GTPase-binding domain (GBD) (Zeng et al., 2003), leading to a conformational change in WASp. In resting cells, WASp exists in a closed, inactive state in which the GBD contacts the verprolin, cofilin, acidic (VCA) region. Cdc42 binding releases the VCA domain, allowing the VCA to interact with Arp2/3 complex (Kim et al., 2000). Phosphorylation of WASp at tyrosine 291 by the Src family kinase Fyn, and subsequent binding of the SH2-SH3 domain module of Src kinases to WASp, works in a synergistic manner with Cdc42 binding to induce optimal WASp activity (Badour et al., 2004a; Torres and Rosen, 2003, 2006). Binding of Nck or the SH3 domain of HS1 (hematopoietic cell-specific lyn substrate 1) or cortactin may also activate WASp or N-WASp (discussed below) (Kowalski et al., 2005; Rohatgi et al., 1999). Analysis of WASp localization in T cells spreading on anti-CD3-coated coverslips reveals that WASp is initially recruited to signaling microclusters. It then translocates to the cell periphery, presumably to aid in actin polymerization in lamellipodial protrusions (Barda-Saad et al., 2005).

WASp-deficient T cells from both mice and humans exhibit consistent defects in signaling pathways leading to IL2 production, but the literature is divided about the importance of WASp for controlling actin polymerization at the IS. Although there are numerous reports that T cells from WAS patients exhibit defective actin responses

(reviewed in (Notarangelo and Ochs, 2003)), Jurkat cells depleted of WASp using RNAi polymerize actin normally (Nolz et al., 2006). Moreover, T cells from two different WASp knockout mice behave differently with respect to their ability to polymerize actin in response to TCR engagement (Badour et al., 2004a; Cannon and Burkhardt, 2004; Krawczyk et al., 2002). A possible explanation for these discrepancies is that other proteins (including the close homologue N-WASp, as well as less closely related proteins such as WAVE2 and HS1) have partially overlapping functions. Indeed, loss of both WASp and N-WASp impairs thymocyte function and development much more severely than loss of either protein alone (Cotta-de-Almeida et al., 2007). Recent work from the Dustin lab supports the idea that WASp-dependent actin polymerization is indispensable for T cell activation (Sims et al., 2007). This study shows that naïve T cells contacting APCs or lipid bilayers containing MHC-peptide complexes and ICAM-1 form a short-lived IS, then migrate some distance before generating a second, more stable contact. Intriguingly, although $WASp^{-/-}$ T cells form an initial contact in this assay, they continue to migrate and fail to re-form and maintain a long-lived IS.

WAVE2

Another important activator of Arp2/3 complex is the WAVE/SCAR family member WAVE2, an NPF that functions as an effector for Rac1. RNAi-mediated suppression of WAVE2 in Jurkat T cells results in loss of actin polymerization at the IS (Nolz et al., 2006; Zipfel et al., 2006). In addition, WAVE2 suppression perturbs conjugate formation, suggesting a role in integrin function. WAVE2 resembles WASp in

that it binds Arp2/3 complex via a C-terminal VCA domain (Figure 1.3), but WAVE2 lacks a GBD and does not bind Rac1 directly (Miki et al., 1998). WAVE2 exists as part of a constitutive complex containing Abi1/2, Sra1/PIR121, Hspc300, IRSp53 and HEM-1 (Billadeau et al., 2007). WAVE complex components stabilize one another against degradation, and suppression of Abi1/2 or HEM-1 also results in actin defects (Innocenti et al., 2004; Miki et al., 2000; Nolz et al., 2006; Zipfel et al., 2006). WAVE complex components also function in WAVE2 targeting and activation. IRSp53 acts as an adaptor between Rac1 and WAVE2, and this interaction leads to the recruitment of WAVE2 to the membrane (Miki et al., 2000). Abi1/2 can mediate an interaction between Abl kinase and WAVE2, resulting in phosphorylation at Tyr 150, which increases the actin polymerization activity of WAVE2 (Leng et al., 2005).

WAVE2-deficient mice have not been generated, due to embryonic lethality (Billadeau et al., 2007). However, a mouse strain with a nonsense mutation in Hem-1 resulting in termination at residue 445 was generated using random mutagenesis (Park et al., 2008). These mice exhibit lymphopenia, neutrophilia, anemia, and pathology throughout a variety of tissues. Thymocyte cellularity is severely reduced, and development is abnormal. Actin responses in neutrophils, macrophages, and T cells from these mice are severely impaired. As expected, this mutation in Hem-1 results in loss of other components of the WAVE complex. Although the mutation responsible may leave a fragment of Hem-1 intact, this study makes it clear that the WAVE complex is integral to leukocyte development and function. It is possible that the function of Hem-1 is

attributable to its role in other complexes (Weiner et al., 2006); however, Hem-1 appears to associate exclusively with the WAVE2 complex in T cells (Nolz et al., 2006).

HS1 and cortactin

A third activator of Arp2/3 complex, and the focus of this dissertation, is HS1. HS1 is a hematopoietic-lineage restricted (Kitamura et al., 1989) homolog of the actin binding protein cortactin (product of the *EMS1* gene). Cortactin is the better studied of the two proteins, and both are discussed below. The domain structures of HS1 and cortactin are shown in Figure 1.3, and a sequence comparison with key features indicated is shown in Figure 1.4.

Like WASp and WAVE, HS1 and cortactin contain an acidic region (NTA) that binds Arp2/3 complex, and both can modestly activate Arp2/3-dependent actin polymerization (Uruno et al., 2001; Uruno et al., 2003b; Weaver et al., 2001). However, HS1 and cortactin also bind to F-actin directly. HS1 binds through its helix-turn-helix (HTH) repeats and coiled-coil (CC) domain (Hao et al., 2005). Cortactin exists as at least two major isoforms, containing either 5.5 or 6.5 HTH repeats; the fourth full repeat is required for F-actin binding (Katsube et al., 2004; Uruno et al., 2001; Weed et al., 2000). Because of their ability to directly bind F-actin, it is thought that HS1 and cortactin function primarily to stabilize existing branched actin filaments by bridging Arp2/3 complex with F-actin (Uruno et al., 2001; Uruno et al., 2003b; Weaver et al., 2001) (Figure 1.5). In keeping with this idea, we have found that HS1-suppressed T cells exhibit unstable actin responses ((Gomez et al., 2006) and Chapter 2). HS1 and cortactin

each contain a proline rich region (PRR) and an SH3 domain at the C-terminus (see below).

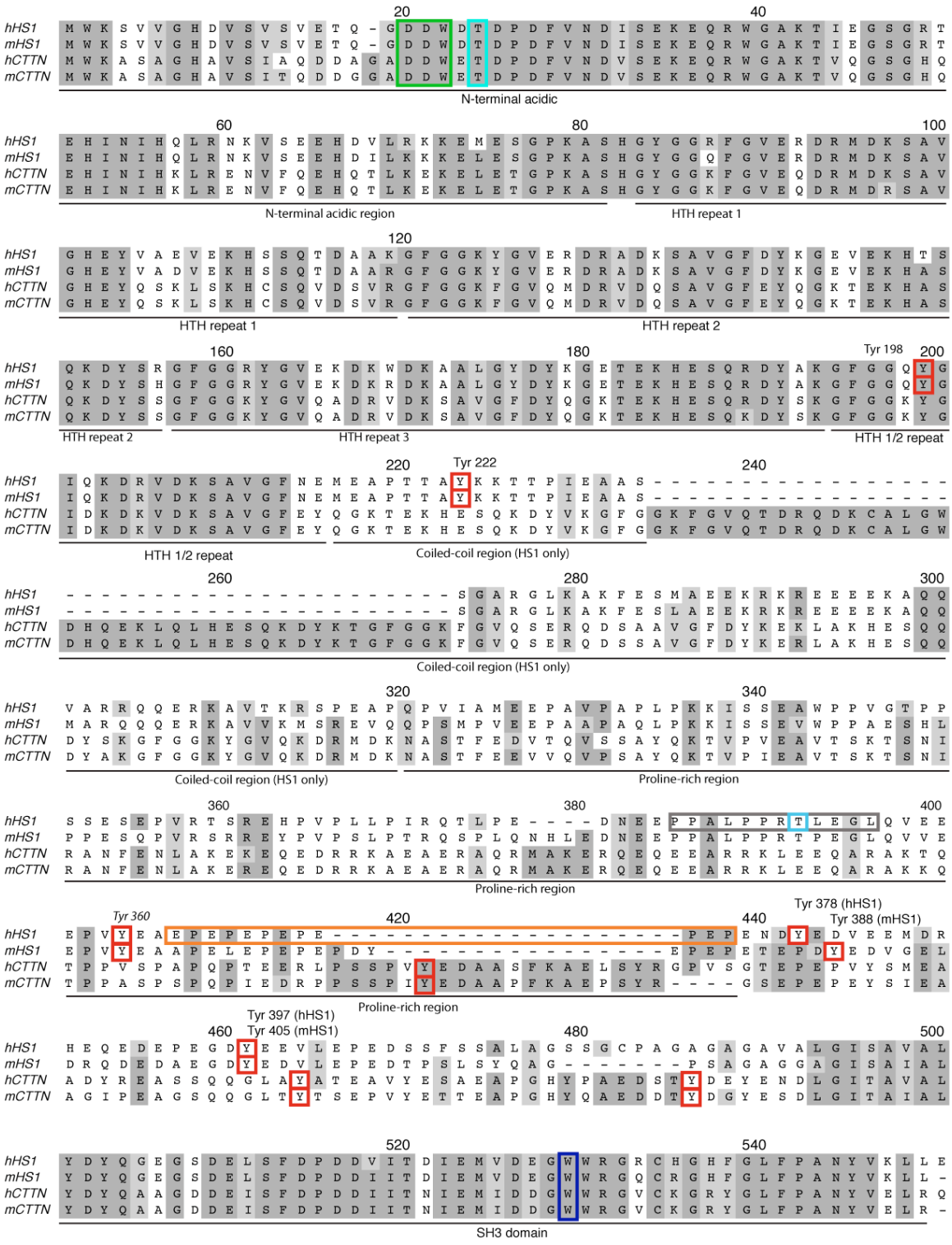
hHS1
mHS1
hCTTN
mCTTN
N-terminal acidic
N-terminal acidic region
HTH repeat 1
HTH repeat 2
HTH repeat 3
HTH 1/2 repeat
Tyr 198
Tyr 222
Coiled-coil region (HS1 only)
Proline-rich region
Tyr 360
Tyr 378 (hHS1)
Tyr 388 (mHS1)
Tyr 397 (hHS1)
Tyr 405 (mHS1)
SH3 domain

Figure 1.4: Sequence alignments of HS1 and cortactin.

Sequence alignments of human HS1 (hHS1), mouse HS1 (mHS1), human cortactin (hCTTN), and mouse cortactin (mCTTN), generated using the multiple sequence alignment function of MacVector, are shown. The cortactin isoforms shown contain 6.5 helix-turn-helix repeats. Sequence identities are shaded in dark gray and sequence similarities are shaded in light gray. Critical residues for Arp2/3 binding, DDW, are boxed in green. The glutamic acid-proline (EP) repeat region in human HS1 is boxed in orange. The tryptophan residue responsible for SH3 domain specificity is boxed in blue. Tyrosine phosphorylation sites that have been empirically identified for the protein of either species are boxed in red and noted above or below the alignment. A poly-proline region that may be involved in intramolecular SH3 domain binding is boxed in gray. A potential MAP kinase recognition site is boxed in light blue. Major domains for HS1 are noted below the alignment. Residue numbering above sequences refers to human cortactin.

Genbank accession numbers of sequences used: hHS1 – AAH16758.1, mHS1

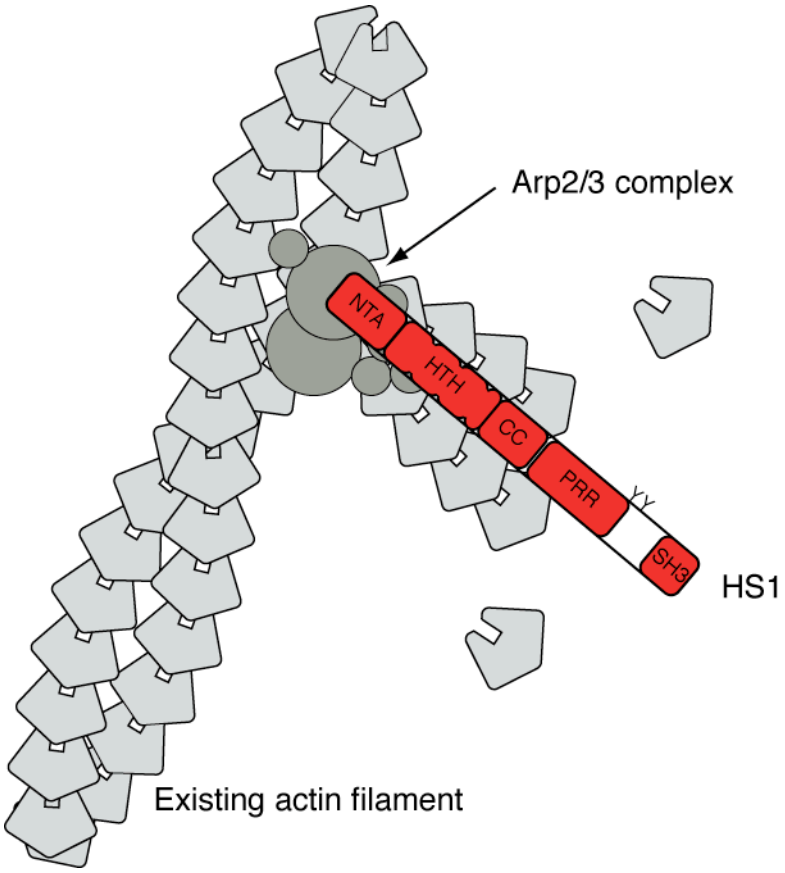

Figure 1.5: Model for HS1 interaction with Arp2/3 complex.

HS1, through its NTA region, interacts with Arp2/3 complex and crosslinks new actin filaments by binding via the HTH repeats and CC region. Model proposed by (Hao et al., 2005).

Interestingly, the repeat region has been used to trace the evolutionary origin of these molecules, as it is a unique feature (van Rossum et al., 2005). Cortactin homologs are found in metazoans, but not in unicellular organisms or in plants. In contrast, HS1 expression is limited to vertebrates, and is thought to have originated from a gene duplication event.

Both HS1 and cortactin are inducibly tyrosine phosphorylated. Cortactin was initially identified as a Src kinase substrate in transformed cells overexpressing activated Src (Kanner et al., 1990; Wu et al., 1991). At least three Src target sites have been identified: Tyr 421, Tyr 466, Tyr 482 (these numbers refer to murine cortactin, and correspond to Tyr 421, Tyr 470, and Tyr 486 in the human protein). Loss of Csk, a negative regulator of Src kinases, leads to increased tyrosine phosphorylation of cortactin, further implicating Src in cortactin phosphorylation (Thomas et al., 1995).

Src-dependent cortactin tyrosine phosphorylation is physiologically important. Phosphorylation regulates the role of cortactin in cell migration, although the effect may vary depending on the molecular and cellular context. Mutation of the three phosphorylated tyrosines substantially impairs migration in a wound healing assay (Huang et al., 1998). One study suggests that tyrosine phosphorylation impairs the ability of cortactin to activate Arp2/3 complex (Martinez-Quiles et al., 2004), but another study demonstrates that, in the presence of Nck, Src phosphorylation potentiates Arp2/3 complex activation (Tehrani et al., 2007). It is thought that tyrosine phosphorylation of cortactin imparts a conformational change, highlighted by increased susceptibility to cleavage by calpain (Huang et al., 1997b; Perrin et al., 2006).

Early studies showed that HS1 undergoes tyrosine phosphorylation in response to B cell receptor (BCR) (Hata et al., 1994; Yamanashi et al., 1993), T cell receptor (TCR) (Takemoto et al., 1995), Fcε Receptor I (FcεRI) (Fukamachi et al., 1994), FcγRIIIA (Zeng et al., 1995), and IL-5 receptor (Sato et al., 1994) stimulation. *In vitro* phosphorylation experiments have identified Tyr 378 and Tyr 397 (these numbers refer to human HS1, and are equivalent to Tyr 388 and Tyr 405, respectively, in the mouse homolog) as targets of Syk family kinases (Brunati et al., 1995; Gomez et al., 2006), and studies in B cells (Yamanashi et al., 1997), T cells (Gomez et al., 2006), NK cells (Butler et al., 2008), and platelets (Brunati et al., 2005) have confirmed that these sites are phosphorylated, most likely by Syk family kinases, in intact cells. Phosphorylation at Tyr 378 and Tyr 397 creates a binding site for SH2 domains ((Gomez et al., 2006), Chapters 2 and 3). This allows HS1 to act as a cytoskeletal adaptor protein, linking signaling machinery to the actin cytoskeleton. HS1 can also be phosphorylated at Tyr 222 by Src family kinases *in vitro* and in intact platelets (Brunati et al., 1999; Brunati et al., 1995); whether this occurs in T cells is not known. Phosphorylation at this site is contingent on phosphorylation at Tyr 378 and Tyr 397 to create a docking site for Src family kinases (Ruzzene et al., 1996). The functional relevance of phosphorylation at this site has not been explored; however, it lies within the CC domain, and may regulate F-actin binding.

Phosphorylation at Tyr 378 and Tyr 397 is a crucial event for HS1 function. The WEHI-231 mutant line M1 is resistant to BCR-induced activation-induced cell death (AICD), due to low expression of HS1 (Fukuda et al., 1995). While forced expression of

wild-type HS1 can rescue the AICD response in one of this line, expression of a Tyr to Phe mutant at both of these sites fails to rescue the AICD response (Yamanashi et al., 1997). Our own studies have confirmed that phosphorylation at these sites in T cells is physiologically important ((Gomez et al., 2006) and Chapter 2).

There is also evidence that HS1 can also be tyrosine phosphorylated at other sites. The c-Abl tyrosine kinase can phosphorylate HS1 *in vitro*, and is required for efficient HS1 phosphorylation in Jurkat cells (Huang et al., 2008). Although the target site is not known, Tyr 421 in cortactin is an Abl target (Boyle et al., 2007), and the HS1 homolog, Tyr 360, is a consensus site for Abl-mediated phosphorylation. Phosphorylation at Tyr 198 has been identified in a proteomic screen in Jurkat cells stimulated with anti-CD3 and anti-CD4 (Salomon et al., 2003), although the physiological relevance of phosphorylation at this site has not been determined. Interestingly, this tyrosine is conserved in HS1 and cortactin, and lies within the half HTH repeat in HS1 (Figure 1.3 and Figure 1.4), suggesting that it may regulate F-actin binding.

Although most work on HS1 phosphorylation has focused on antigen receptor signaling, ligation of other cell-surface molecules may induce HS1 phosphorylation or enhance antigen receptor-induced phosphorylation. Ligation of the costimulatory receptors CD2 and CD28 alone or in conjunction with the TCR does not affect total HS1 tyrosine phosphorylation (Hutchcroft et al., 1998). In contrast, a recent study from Cooper and colleagues has shown that, in human NK cells plated on integrin ligands, HS1 undergoes tyrosine phosphorylation, an event that is required for adhesion (Butler et al., 2008).

Interestingly, WASp, WAVE2 and HS1 are highly modular in structure and can interact with multiple signaling molecules at the IS. This structure undoubtedly facilitates coordination of actin polymerization. Cortactin can cooperate with various combinations of WIP, WASp, and Nck1 to activate Arp2/3 complex (Tehrani et al., 2007), and we have explored the possibility that HS1 participates in similar interactions ((Gomez et al., 2006) and Chapter 2). In addition to the actin-regulatory domains described above, HS1 and cortactin contain a proline-rich region and an SH3 domain at the C terminus. The SH3 domain of cortactin has been shown to enhance N-WASp-dependent actin polymerization, at least *in vitro* (Martinez-Quiles et al., 2004).

It is also likely that HS1, as well as WASP and WAVE2, facilitate T cell activation by coordinating other signaling molecules independently of their role in actin polymerization. For example, the SH3 domain of the Src family kinase Lck (leukocyte-specific protein tyrosine kinase) binds to the proline-rich region of HS1 constitutively (Takemoto et al., 1995), and the SH2 domain of Lck interacts with HS1, probably through Tyr 378 and Tyr 397 (Gomez et al., 2006). This interaction may permit phosphorylation at Tyr 222 by Src family kinases *in vitro* and in intact platelets (Brunati et al., 2005; Brunati et al., 1999; Ruzzene et al., 1996). However, the functional significance of this interaction is unknown.

Although the function of the HS1 SH3 domain has not been elucidated, the SH3 domain of cortactin interacts with dynamin family members and is important for endocytosis (McNiven et al., 2000; Sauvonnet et al., 2005). The SH3 domain of cortactin

also interacts with WIP and WASp or N-WASP and can potentiate actin polymerization through that pathway (Kinley et al., 2003; Martinez-Quiles et al., 2004).

In addition to activation by tyrosine phosphorylation, HS1 and cortactin may be regulated by serine/threonine phosphorylation. Phosphorylation of cortactin at Ser 405 and Ser 418 promotes actin polymerization (Martinez-Quiles et al., 2004). This may occur by relieving an intramolecular interaction between the SH3 domain and the proline-rich region. HS1 also undergoes serine/threonine phosphorylation, although the sites are unknown (Ruzzene et al., 2000). Although HS1 lacks homologs to cortactin Ser 405 and Ser 418, HS1 does have a potential MAP kinase recognition site at Thr 352 (Figure 1.4) (Takemoto et al., 1995). HS1 becomes a substrate of caspase 3 upon induction of apoptosis (Chen et al., 2001), and serine/threonine phosphorylation by CK2 renders HS1 resistant to caspase 3-mediated cleavage (Ruzzene et al., 2002). Although the physiological relevance of this phenomenon is unclear, it suggests that phosphorylation induces a conformational change, as described above for cortactin.

No crystal structures for HS1 or cortactin have been published, presumably because conformational flexibility of the HTH repeat region makes the protein difficult to crystallize (Pant et al., 2006). However, low-resolution studies have been conducted on the cortactin. Electron microscopy experiments have suggested that cortactin exists as a relatively long, unstructured molecule, with a length of approximately 220-290 Å (Weaver et al., 2002). In contrast, a more recent study using a combination of small angle X-ray scattering, circular dichroism, and chemical crosslinking suggested an ordered, globular structure with a length of approximately 110 Å (Cowieson et al., 2008).

In this model, cortactin folds in such a way that the SH3 domain is close to the HTH repeats; the authors do not exclude an interaction with the PRR. The differences between these two structures may reflect a conformational change that cortactin may undergo, from a closed, globular state to a more open, unstructured conformation.
However, the differences between the two structures may also be partially attributable to the differences in the methodologies between the two studies. To date, no such structural studies on HS1 have been published, but its structure is likely to bear some resemblance to that of cortactin. Differences between the conformations of the molecules, in particular from the differences in the HTH repeat region, may reflect the differences in the signaling machinery between hematopoietic and non-hematopoeitic cells.

HS1 knockout mice

Mice lacking HS1 have been described (Taniuchi et al., 1995). These mice develop normally, and no overt pathology has been detected. A limited analysis of B cell and T cell development revealed normal progression through developmental stages, and peripheral lymphoid populations appeared to be normal. However, responses to antigen receptor ligation, for both B cells and T cells, were blunted: proliferative responses were reduced, peritoneal B cells were abnormally resistant to activation-induced cell death (AICD) resulting from multivalent BCR crosslinking, and thymocytes exhibited a defect in negative selection in an H-Y transgenic model system. The phenotype of T cells from these mice is further explored in Chapter 4.

HS1 and cortactin in disease

Both HS1 and cortactin have been linked to disease. A variant of HS1 that lacks the third HTH repeat, due to an exon-skipping mutation, was found in a SLE (systemic lupus erythematosus) patient, as well as in the patient's father and a sister, who also displayed anti-nuclear antibodies (Sawabe et al., 2003); henceforth, this variant is referred to as $HS1^{HTH2.5}$. Moreover, an allelic variant with eight glutamic acid-proline repeats (referred to as $HS1^{EP8}$) instead of the more common six ($HS1^{EP6}$) within the proline-rich region (Figure 1.4), is significantly more common in SLE patients than in the healthy population (Otsuka et al., 2004). 18.4% of SLE patients examined in this study were homozygous for $HS1^{EP8}$, whereas 9.8% of healthy individuals had this genotype. In these studies, expression of $HS1^{HTH2.5}$ or $HS1^{EP8}$ promoted AICD more strongly than did $HS1^{EP6}$ in the WEHI231 cell death assay described above. The reason for this has not been explored.

Numerous studies have linked cortactin expression to malignancy. Cortactin overexpression has been associated with higher metastatic potential and poor prognoses in many cancers, including breast, colorectal, and gastric cancers (Weaver, 2008). The role of cortactin in cancer most likely stems from its effects on cell migration. It is a component of invadopodia, actin-rich membrane protrusions that allow migrating cells to invade tissues by degrading extracellular matrix (Bowden et al., 1999). Additionally, a switch in expression from HS1 to cortactin has been found in certain B lymphoid tumor lines (Miglarese et al., 1994). The reason for this switch is unclear; however, cortactin is a more potent activator of Arp2/3 complex than HS1, and thus may be a more potent

promoter of tumor metastasis. However, HS1 has also been linked to malignancy (Scielzo et al., 2005). Constitutive phosphorylation of HS1 in chronic lymphocytic leukemia is associated with a significantly shorter survival time.

Cortactin has also been shown to play a role in bacterial invasion at the level of pathogen uptake and trafficking. Several studies have demonstrated that cortactin is involved in cell invasion by intracellular bacteria, including *Shigella flexneri*, a causative agent of dysentery (Bougneres et al., 2004; Dehio et al., 1995; Dumenil et al., 1998); *Staphylococcus aureus* (Agerer et al., 2005)*; Neisseria meningitidis*, which can cause life-threatening meningitis (Hoffmann et al., 2001); and food-borne pathogens *Salmonella typhimurium* and *Listeria monocytogenes* (Barroso et al., 2006; Unsworth et al., 2004; Veiga and Cossart, 2005) (reviewed in (Selbach and Backert, 2005)). Although no such role has been demonstrated for HS1, HIV depends on intact T cell cytoskeletal dynamics for proper assembly (Jolly et al., 2007). It would be interesting to determine whether HS1 deficiency imparts increased resistance to pathogen invasion.

The various disease associations of both HS1 and cortactin highlight the importance of understanding the basic biology of these proteins and the broader immunological and cell biological processes in which they are involved.

INTERPLAY BETWEEN T CELL SIGNALING AND ACTIN DYNAMICS

TCR signaling to and through the actin cytoskeleton

TCR engagement induces a tyrosine phosphorylation cascade that facilitates the assembly

of the signaling machinery into multimolecular signaling complexes (Smith-Garvin et al., 2009). One of the earliest events after TCR engagement is the activation of the Src family kinase Lck, which phosphorylates ITAMs (immunotyrosine-based activation motifs) within the TCR complex. This creates a docking site for the Syk family kinase ZAP-70 (ζ-chain associated protein kinase-70 kDa). ZAP-70, in turn, phosphorylates the adapter proteins LAT (linker for activation of T cells) and Slp-76 (SH2-domain containing leukocyte protein of 76 kDa). Slp-76 and LAT nucleate the assembly of large multimolecular complexes, often referred to as signalosomes. Although the specific composition is likely to vary between individual complexes, signaling proteins that participate include PLCγ1 (phospholipase C γ1), the Rho GEF (guanine nucleotide exchange factor) Vav-1, and the Tec family kinase Itk (IL2-inducible T cell kinase) (Figure 1.6). Other adaptors, including Nck (non-catalytic region of tyrosine kinase), Grb2, and GADS are also involved.

These proteins stabilize one another at sites of TCR engagement, and loss of any of these molecules results in disruption of actin polymerization at the IS (Bubeck Wardenburg et al., 1998; Bunnell et al., 2001; Fischer et al., 1998). Interaction with complex components promotes the activation of Vav1 by phosphorylation and phosphoinositide-dependent engagement of its PH domain (Bustelo, 2002). Vav1 then generates localized pools of active Cdc42 and Rac1, promoting actin polymerization by WASp and WAVE2, respectively (Dombroski et al., 2005; Labno et al., 2003). As discussed above, other events also contribute to WASp and WAVE2 activation; however, Vav1 function is clearly critical (Fischer et al., 1998; Holsinger et al., 1998).

Importantly, Vav1 binds to multiple actin regulatory molecules, suggesting that its central role in controlling actin dynamics involves adaptor functions as well as GEF activity.

Itk plays a key role in T cell activation, regulating both cytoskeletal rearrangements and Ca^{2+} flux. The domain structure of Itk is shown in Figure 1.6. Itk contains a pleckstrin homology domain that mediates inducible binding to PtIns(3,4,5)P_3 generated by PI3K (phosphoinositide 3-kinase) upon T cell activation, a proline-rich region, an SH3 domain, an SH2 domain, and the kinase region. The proline rich region, SH3 domain, and SH2 domain are involved in intermolecular interactions with other signalosome components (Bunnell et al., 2000).

Although it lacks domains that interact directly with the actin cytoskeleton, Itk is required for actin polymerization at the IS. T cells from $Itk^{-/-}$ mice or Jurkat cells depleted of Itk by RNAi exhibit severe defects in actin polymerization at the IS (Dombroski et al., 2005; Grasis et al., 2003; Labno et al., 2003). Interestingly, control of actin dynamics is an adaptor function of Itk, since an intact SH2 domain, but not kinase activity, is required. Itk regulates cytoskeletal dynamics in part by recruiting Vav1 to the IS. The link between Itk and the actin cytoskeleton is further explored in Chapter 3.

Interactions between signalosome components, as well as interactions between the SH3 domain and proline rich region of Itk (in *cis* or in *trans*) are important for regulating its kinase activity. Phosphorylation of Itk at Tyr 180 and Tyr 511 induces conformational changes that result in full activation of kinase activity (Joseph et al., 2007; Wilcox and Berg, 2003). Binding of Slp-76 to the SH2 and SH3 domain of Itk promotes kinase activity by preserving the open, active conformation (Bogin et al., 2007). Upon

activation, Itk phosphorylates PLCγ1 at activating tyrosines (Bogin et al., 2007), leading to Ca^{2+} and DAG signaling, discussed below.

Costimulation-dependent actin regulatory pathways

Costimulatory signaling also controls actin responses at the IS (Tskvitaria-Fuller et al., 2003). A major consequence of costimulation via CD28 is the activation of PI3K. Through the production of PtIns(3,4,5)P_3, PI3K can influence the localization and activation of PH-domain containing signaling molecules including Vav1 and Itk, leading to enhanced and/or sustained activation of the pathways described above (Finkelstein and Schwartzberg, 2004; Han et al., 1998; Michel et al., 2000). CD28 can also signal through a PI3K-Ras pathway to dephosphorylate and activate cofilin, an actin filament severing protein (Wabnitz et al., 2006). Though less well studied, costimulatory signaling via other molecules such as CD2, CD82, CD46 and ICOS also affects TCR-induced actin responses (Delaguillaumie et al., 2002; Nukada et al., 2006; Wabnitz et al., 2007; Zaffran et al., 2001). Thus, one way that costimulation promotes full T cell activation is by augmenting actin-dependent signaling events at the IS.

Pathways leading to Ca^{2+} signaling in T cells

A common consequence of disruption of actin dynamics in T cells is perturbation in Ca^{2+} signaling events. Ca^{2+} signaling pathways downstream of TCR engagement are therefore summarized here, starting from phosphorylation and activation of PLCγ1.

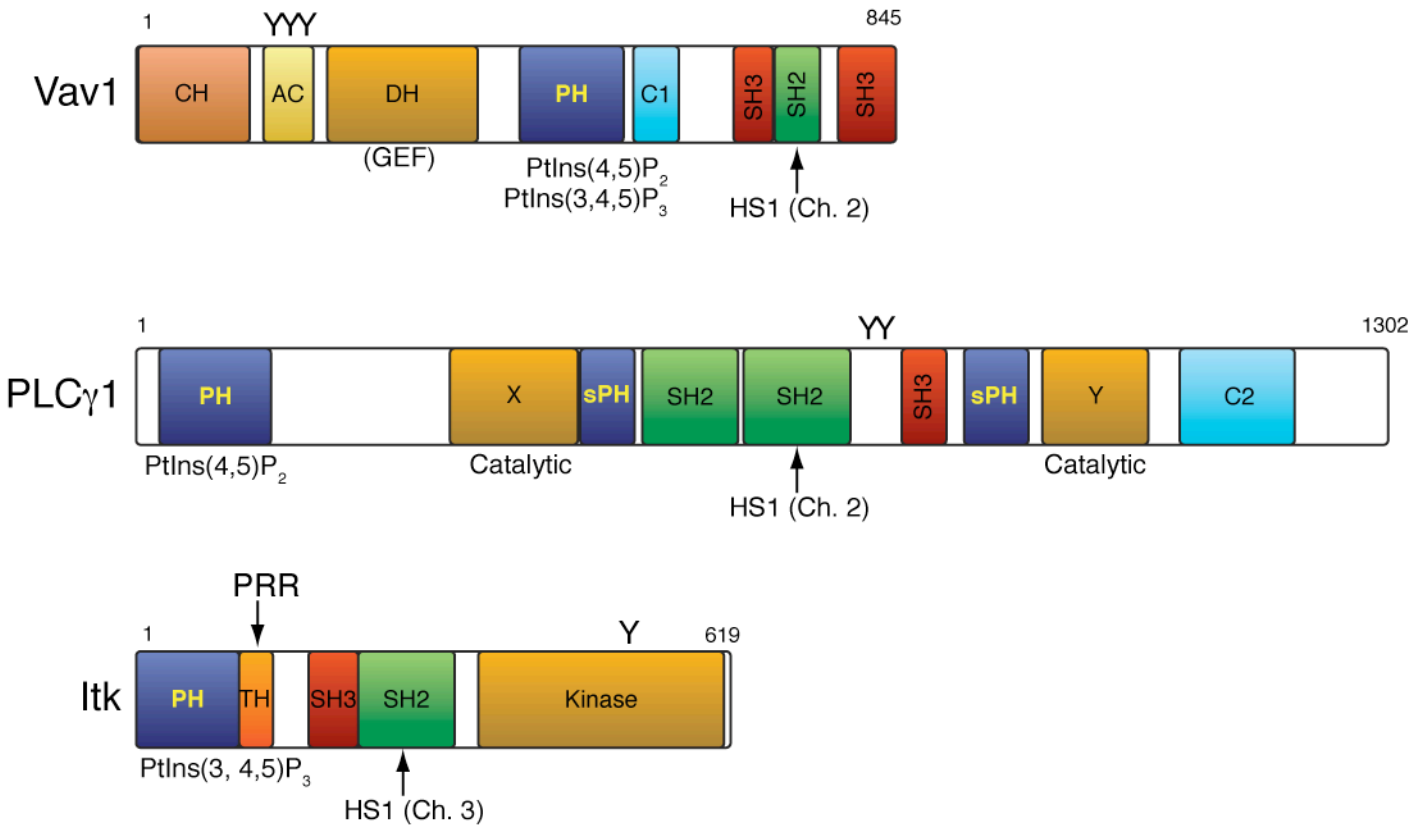
Vav1
1
YYY
845
CH
AC
DH
PH
C1
SH3
SH2
SH3
(GEF)
PtIns(4,5)P2
PtIns(3,4,5)P3
HS1 (Ch. 2)
PLCγ1
1
YY
1302
PH
X
sPH
SH2
SH2
SH3
sPH
Y
C2
PtIns(4,5)P2
Catalytic
Catalytic
HS1 (Ch. 2)
PRR
Itk
1
Y
619
PH
TH
SH3
SH2
Kinase
PtIns(3, 4,5)P3
HS1 (Ch. 3)

Figure 1.6: Domain structures of signaling molecules of interest.

The domain structure of key signaling molecules is shown, with related regions colored similarly. Important binding partners and targets of catalytic activity are listed below the relevant domains. Key tyrosine phosphorylation sites (Y) are indicated, and total amino acid numbers are shown at the top right of each.

Abbreviations: CH - calponin homology; Ac – acidic; DH – Dbl homology; PH – Pleckstrin homology; sPH – split PH; SH2 – Src homology 2; TH – Tec homology

PLCγ1 is a key signaling protein responsible for translating the tyrosine phosphorylation cascade and signalosomes assembly events into Ca^{2+} flux responses that lead to transcription factor activation. TCR engagement leads to rapid (within one minute) tyrosine phosphorylation of PLCγ1. Although it contains numerous tyrosine phosphorylation sites, Tyr 775 and Tyr 783 are considered most important: phosphorylation at both sites is required for full activation (Serrano et al., 2005) (by convention, PLCγ1 residues are numbered according to their position within the bovine protein).

PLCγ1 is a large (145 kDa) protein composed of a pleckstrin homology (PH) domain followed by an EF hand at the amino terminus (Figure 1.6). The catalytic region is divided into the X and Y fragments, which are situated N-terminal and C-terminal, respectively, to a segment containing two SH2 domains and one SH3 domain. A C2 domain is located at the C-terminus of the molecule.

The split nature of the catalytic region and presence of multiple Src homology domains underscore the importance of regulation by protein conformation and intra- and intermolecular interactions. Recruitment of PLCγ1 to the membrane and to signaling microclusters requires its N-terminal SH2 domain, which interacts with phosphorylated Tyr 132 of LAT (Braiman et al., 2006; Stoica et al., 1998; Zhang et al., 2000). Paradoxically, this domain appears not to be necessary for tyrosine phosphorylation of PLCγ1 (Braiman et al., 2006) or downstream transcription factor activation by PLCγ1 (Irvin et al., 2000), although these requirements may vary depending on the experimental system. In contrast, the C-terminal SH2 domain is required for enzymatic activity, but

not for recruitment to microclusters or for phosphorylation at Tyr 783 (Braiman et al., 2006; Poulin et al., 2005). In keeping with this, phosphorylation at Tyr 783 is insufficient to activate lipase activity; additional events are required (Sekiya et al., 2004). Phosphorylation at Tyr 783 can activate PLCγ1 by creating a binding site for the C-terminal SH2 domain. This intramolecular interaction induces a conformational change that causes the X and Y domains to assemble into a fully functional catalytic region (Poulin et al., 2005). Intermolecular interactions can also activate PLCγ1, most likely by inducing conformational changes: binding of SHIP1 to the SH3 domain results in increased Ins(1,4,5)P_3 production in transfected COS7 cells (Song et al., 2005).

Several studies have demonstrated that PLCγ1 interacts with components of the actin cytoskeleton. In rat embryo fibroblasts, immunofluorescence microscopy studies have shown an SH3 domain-dependent localization of PLCγ1 to the actin cytoskeleton (Bar-Sagi et al., 1993). Several studies have biochemical approaches to show that PLCγ1 or PLCγ2 inducibly interact with F-actin in a manner dependent on either SH2 domain (Dearden-Badet and Mouchiroud, 2005; Nojiri and Hoek, 2000; Pei et al., 1996). Although the physiological relevance of this cytoskeletal interaction is unclear, poisoning of actin dynamics with CytoD could impair PLCγ1 activity (Suzuki and Takahashi, 2001). No such studies have been conducted in T cells; however, pharmacological disruption of actin dynamics or loss of actin regulatory proteins in T cells commonly disrupts Ca^{2+} signaling, as discussed above. We have shown that the C-terminal SH2 domain of PLCγ1 interacts with HS1 ((Gomez et al., 2006) and Chapter 2). This may

serve to regulate PLCγ1 localization and/or function by linking it to the actin cytoskeleton. This idea is explored in chapter 3.

Upon activation, PLCγ1 hydrolyzes the membrane lipid PtIns(4,5)P_2 (phosphatidylinositol 4,5 bisphosphate) to generate Ins(1,4,5)P_3 (inositol 1,4,5 trisphosphate) and DAG (diacylglycerol). Ins(1,4,5)P_3 binds to Ins(1,4,5)P_3 receptors on the ER (endoplasmic reticulum), inducing the release of Ca^{2+} (calcium ions) from ER stores (Majerus et al., 1986; Rhee, 2001; Singer et al., 1997). Depletion of ER Ca^{2+} stores induces a conformational change in the Ca^{2+} sensor STIM1 (stromal interacting molecule 1) that allows it to interact with and activate CRAC (Ca^{2+} release-activated Ca^{2+}) channels (Luik et al., 2006; Park et al., 2009; Wu et al., 2006). Although electrophysiological experiments had permitted functional characterization of the CRAC channel, its molecular identity was not known until three groups independently identified Orai1 as a critical component of CRAC channels (Feske et al., 2006; Prakriya et al., 2006; Vig et al., 2006; Zhang et al., 2006).

Activation of CRAC channels causes the influx of Ca^{2+} from the extracellular milieu (Oh-hora and Rao, 2008). The increase in intracellular Ca^{2+} is detected by the calcium binding protein calmodulin. Ca^{2+} binding causes calmodulin to interact with and activate the phosphatase calcineurin, which dephosphorylates cytosolic NF-AT (nuclear factor of activated T cells) family transcription factors. This reveals the nuclear localization sequence of NF-AT, allowing it to translocate to the nucleus to drive gene expression.

While significant progress has been made in understanding the upstream events linking TCR engagement to actin remodeling, our understanding of how actin contributes to downstream signaling events is largely restricted to activation of the IL2 promoter. Disruption of the actin network perturbs the activation of each of the three key elements within the *IL2* promoter (NF-AT, NFκB, and AP-1). Virtually every condition that affects actin dynamics affects Ca^{2+} signaling pathways leading to NF-AT activation. Treatment of T cells with CytoD affects Ca^{2+} flux and NF-AT activation, though as noted above, the effects vary depending on the time of treatment and dose of inhibitor (Holsinger et al., 1998; Rivas et al., 2004; Valitutti et al., 1995). Diminished Ca^{2+} influx and inefficient NF-AT activation have also been observed in T cells deficient for Vav1, WASp, WAVE2 and HS1 (Cannon and Burkhardt, 2004; Cianferoni et al., 2005; Fischer et al., 1998; Gomez et al., 2006; Holsinger et al., 1998; Nolz et al., 2006; Zhang et al., 1999). However, the requirement is not simply for actin per se, because individual actin regulatory proteins facilitate Ca^{2+} signaling in distinct ways. For example, WAVE2 deficient T cells exhibit defects in coupling store release to CRAC channel activation (Nolz et al., 2006), while HS1-deficient T cells exhibit a defect at the level of release from stores, as shown in Chapter 3. T cells deficient for Vav1 and WASp also fail to activate AP1, likely stemming from upstream defects in ERK activation (Cianferoni et al., 2005; Costello et al., 1999). Activation of NFκB is defective in T cells lacking Vav1 or HS1 (Costello et al., 1999; Gomez et al., 2006). The molecular basis for this defect has not been established, but it may reflect defects in PKCθ signaling.

Additional analysis of signaling events in T cells lacking actin regulatory proteins will be required to fill in the "black box" linking actin polymerization and changes in T cell gene expression. It is unlikely that these events represent simple, linear pathways. There is growing evidence that actin networks facilitate formation of or stabilize protein-protein interactions to promote sustained signaling (Campi et al., 2005; Gomez et al., 2006). In addition, actin promotes the internalization of TCR signaling complexes and costimulatory molecules, an event that is thought in some cases downregulate signaling, and in others, to sustain it (Badour et al., 2007; McGavin et al., 2001).

This dissertation describes an analysis of the regulation of HS1 and its role in T cell receptor signaling. As shown in Chapter 2, I have found that $HS1^{-/-}$ T cells exhibit profound defects in actin polymerization at the IS in response to TCR engagement, and that these cells exhibit dampened signaling Ca^{2+} signaling, resulting in a defect in IL2 production. Tyr 378 and Tyr 397 were identified as key phosphorylation sites, and HS1 function in T cells depends on the integrity of phosphorylation at these sites. In Chapter 3, I describe an interaction between HS1 and Itk that is required for recruitment of HS1 to the IS. In addition, I explore the role of HS1 in Ca^{2+} signaling, finding that HS1 is important for proper recruitment of PLCγ1 to the actin cytoskeleton and to signaling microclusters. Chapter 4 describes a characterization of T cells from the $HS1^{-/-}$ mouse. Thymocyte and peripheral populations develop normally; however, $HS1^{-/-}$ T cells exhibit defects in T_H1 skewing, likely a result of defects in IFNγ production. This may reflect a defect in Ca^{2+} signaling. Finally, in Chapter 5, I discuss the implications of these findings and propose future avenues for research.

Chapter 2: HS1 functions as an essential actin-regulatory adapter protein at the Immune Synapse[2]

Summary

HS1, the leukocyte-specific homologue of cortactin, regulates F-actin in vitro and is phosphorylated in response to TCR ligation, but its role in lymphocyte activation has not been addressed. We demonstrate that HS1-deficient T cells fail to accumulate F-actin at the immune synapse (IS), and upon TCR ligation form actin-rich structures that are disordered and unstable. Early TCR activation events are intact in these cells, but Ca^{2+} influx and IL2 gene transcription are defective. Importantly, HS1 tyrosine phosphorylation is required for its targeting to the IS, and for its function in regulating actin dynamics and IL2 promoter activity. Phosphorylation also links HS1 to multiple signaling proteins, including Lck, PLCγ1, and Vav1, and is essential for the stable recruitment of Vav1 to the IS. Taken together, our studies show that HS1 is indispensable for signaling events leading to actin assembly and IL2 production during T cell activation.

Introduction

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[2] This chapter is a modified version of a manuscript titled, "HS1 functions as an essential actin-regulatory adapter protein at the immune synapse." It was published in the June 2006 Issue of Immunity (volume 24, page 741) with the following author list: Timothy S. Gomez, Sean D. McCarney, Esteban Carrizosa, Christine M. Labno, Erin O. Comiskey, Jeffrey C. Nolz, Peimin Zhu, Bruce D. Freedman, Marcus R. Clark, Daniel D. Billadeau, and Janis K. Burkhardt

In response to interaction with APCs, T cells undergo dramatic shape changes to form a flattened contact site enriched in F-actin and signaling molecules, termed the immune synapse (IS) (Bromley et al., 2001; Kupfer and Kupfer, 2003). Actin polymerization at the IS stabilizes conjugate formation and facilitates T cell activation (Fuller et al., 2003). Several proteins have been implicated in regulating actin dynamics at the IS, including the proximal tyrosine kinases Lck and ZAP-70 and the adaptors LAT and SLP-76 (Bubeck Wardenburg et al., 1998; Bunnell et al., 2001; Morgan et al., 2001; Zeng et al., 2003). In addition, the Rho family guanine nucleotide exchange factor Vav1 regulates actin responses through the small GTPases Cdc42 and Rac1 (Turner and Billadeau, 2002; Zeng et al., 2003). Finally, we recently showed that the Tec kinase Itk and the large GTPase Dynamin 2 collaborate with Vav1 to control actin responses at the IS (Dombroski et al., 2005; Gomez et al., 2005; Labno et al., 2003). While much has been learned about upstream signaling pathways, the immediate effectors controlling actin dynamics are still poorly understood. The two Rho family proteins regulated by Vav1, Rac1 and Cdc42, both play pivotal roles. Rac1 activates the WAVE2 complex, which we recently showed is required for actin-dependent signaling at the IS (Nolz et al., 2006), while Cdc42 regulates Wiskott-Aldrich Syndrome protein (WASp) (Cannon et al., 2001). Although WASp activation is required for T cell activation (Badour et al., 2004b), recent experiments indicate that WASp-deficient T cells can retain the ability to polymerize actin at the IS (Cannon and Burkhardt, 2004). Thus, other mechanisms must contribute to the regulation of actin dynamics at the IS.

Hematopoietic lineage cell-specific protein 1 (HS1, HCLS1, LckBP1) is a major substrate for tyrosine phosphorylation during T and B cell activation (Ruzzene et al., 1996; Takemoto et al., 1996; Yamanashi et al., 1993). HS1, which is expressed only in hematopoietic cells, is related to cortactin (EMS1), a widely expressed oncogene that was recently identified as an actin regulatory protein (Daly, 2004). Similarly to cortactin, HS1 contains an Arp2/3 complex binding domain, a region of 37 amino acid tandem repeats and a coil-coiled region that bind F-actin (Hao et al., 2005), a proline rich domain that binds to Lck (Takemoto et al., 1996), and a C terminal SH3 domain. *In vitro* studies show that HS1 and cortactin can activate Arp2/3-dependent actin polymerization and prolong the half-life of branched actin structures (Uruno et al., 2003a; Weaver et al., 2002; Weaver et al., 2001). $HS1^{-/-}$ mice display defects in antigen-induced clonal expansion and lymphocyte deletion (Taniuchi et al., 1995), but the role of HS1 in T cell signaling has not been tested. Here we demonstrate that HS1 is required for sustained actin responses at the IS and Ca^{2+} signaling events leading to IL2 gene expression.

Results

HS1 regulates the accumulation of F-actin at the immune synapse

We began our analysis by examining HS1 distribution in T cells responding to APCs. HS1 co-localized with F-actin at the IS in conjugates formed with Jurkat T cells, primary human $CD4^+$ T cells, and murine DO11.10 TCR Tg T cells (Figure 2.1A). Recruitment of HS1 to the cell-cell contact site was antigen-dependent, as was the

accumulation of F-actin (data not shown and Figure 2.1E). Thus, T cell activation induces recruitment of HS1 to the IS, where it co-localizes closely with F-actin.

To test HS1 function, we suppressed HS1 expression in Jurkat T cells using shRNA-mediated gene silencing. Two different HS1 targeting vectors (shHS1b and shHS1f), but not empty vector or a scrambled version of shHS1b (shHS1mut), suppressed HS1 protein to 10% of control or less (Figure 2.1B). All studies were performed using both shHS1b and shHS1f, with similar results. HS1-suppressed T cells formed conjugates efficiently with SEE-pulsed B cells (Figure 2.1C). To assess actin responses, conjugates were fixed at 15 minutes and analyzed by microscopy. As expected, control transfectants showed accumulation of HS1 and F-actin at the IS (Figure 2.1D). HS1-suppressed cells no longer stained for HS1, verifying that HS1 was efficiently depleted. Although these cells flattened relatively normally against the APCs, F-actin labeling at the IS was absent or greatly reduced. Time-course analysis revealed that HS1-suppressed cells initially polymerized actin efficiently (Figure 2.1E), but failed to maintain F-actin at the IS, such that by 5 minutes, the response was reduced to background levels (defined by control cells without SEE).

To validate these findings, we analyzed primary lymph node T cells from $HS1^{-/-}$ mice responding to P815 cells decorated with anti-TCR antibodies. Wild type T cells formed a well-defined F-actin-rich IS in this assay (Figure 2.1F). In contrast, although T cells from $HS1^{-/-}$ mice formed conjugates with P815 cells efficiently, they failed to exhibit clear F-actin polymerization at the IS. After 30 minutes, the frequency of $HS1^{-/-}$ T cells showing F-actin labeling at the IS was at baseline levels (Figure 2.1G).

Moreover, this defect was not restored by the addition of costimulatory antibody. Thus, we conclude that HS1 is required for the stable accumulation of F-actin at the IS.

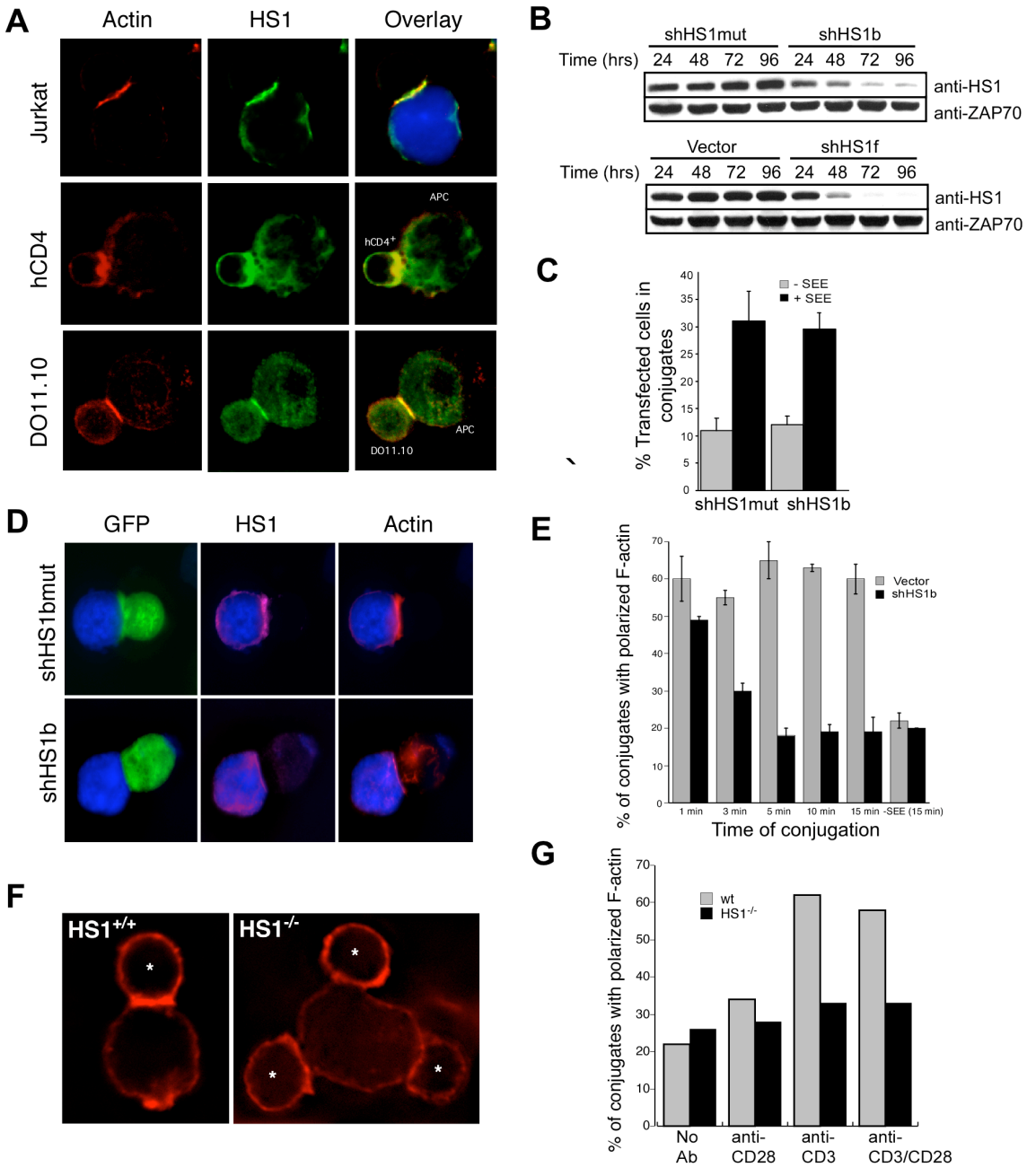
A
Actin
HS1
Overlay
Jurkat
hCD4
DO11.10
APC
hCD4+
APC
DO11.10
B
shHS1mut
shHS1b
Time (hrs) 24 48 72 96 24 48 72 96
anti-HS1
anti-ZAP70
Vector
shHS1f
Time (hrs) 24 48 72 96 24 48 72 96
anti-HS1
anti-ZAP70
C
% Transfected cells in conjugates
- SEE
+ SEE
shHS1mut shHS1b
D
GFP
HS1
Actin
shHS1bmut
shHS1b
E
% of conjugates with polarized F-actin
Vector
shHS1b
1 min 3 min 5 min 10 min 15 min -SEE (15 min)
Time of conjugation
F
HS1+/+
HS1-/-
G
% of conjugates with polarized F-actin
wt
HS1-/-
No Ab
anti-CD28
anti-CD3
anti-CD3/CD28

Figure 2.1: HS1 regulates the accumulation of F-actin at the IS

A, Conjugates were formed between Jurkat T cells and SEE-pulsed NALM6 B cells, primary human $CD4^+$ T cells and sAg-pulsed Raji B cells, or 2° DO11.10 Tg T cells and ova-pulsed A20 B cells. Conjugates were labeled with anti-HS1 and phalloidin to label F-actin. B cells were labeled with CMAC (blue and not shown). *B,* Short hairpin RNA (shRNA) suppression time-course for HS1. Jurkat cells were transiently transfected with RNA targeting vectors (shHS1b or shHS1f), a mutated form of shHS1b (shHS1mut), or vector control. Lysates were prepared at the indicated times post-transfection and immunoblotted for HS1 and ZAP70. *C*, To assess conjugation efficiency, Jurkat cells were transfected with shHS1b or shHS1mut control vectors containing a separate GFP cassette. T cells were incubated with SEE-pulsed, PKH26-stained EBV-B cells and the % transfected T cells in conjugates was determined by flow cytometry. *D,* To assess actin responses at the IS, Jurkat cells transfected as in C (GFP) were incubated with SEE-pulsed NALM6 B cells (blue). Fixed conjugates with labeled with phalloidin to visualize F-actin and anti-HS1. *E*, Conjugates formed as in D were fixed at the indicated times following initial contact and labeled with phalloidin. Conjugates containing GFP^+ T cells were scored for F-actin at the IS. *F*, WT and $HS1^{-/-}$ T cells (∗) were conjugated to P815 cells bearing anti-CD3 antibodies and stained with phalloidin (red). *G*, Conjugates were formed between $HS1^{+/+}$ or $HS1^{-/-}$ T cells and P815 cells bound with the indicated stimulating antibodies and scored for F-actin at the IS. Data are from one representative experiment. Bars in C and E represent mean ±StDev from three experiments.

HS1-deficient cells fail to stabilize actin following TCR engagement

In non-hematopoietic cells, the HS1 homologue cortactin is thought to stabilize F-actin (Daly, 2004). We confirmed that neither Jurkat cells nor primary mouse T cells expressed detectable levels of cortactin mRNA (Figure 2.2), and we observed no compensatory upregulation of cortactin in $HS1^{-/-}$ T cells or HS1-suppressed Jurkat cells. To ask if HS1 functions by stabilizing F-actin, we analyzed the spreading of Jurkat cells stably expressing GFP-actin on anti-TCR-coated coverslips (Bunnell et al., 2001). Control cells spread in a highly ordered fashion, forming a round lamellipodial structure with uniform width (Figure 2.3A and Supplemental Movies 2.1-2.3). Spreading was typically maximal by 2 minutes, with retraction after about 5 minutes. DIC images (not shown) showed ruffling at the cell periphery and radial retrograde flow within the lamellar region; this process is also evident in the GFP-actin videos (Supplemental Movies 2.4-2.7). In contrast, HS1-suppressed cells spread erratically. These cells continuously sent out actin-rich protrusions, but these were asymmetric and quickly retracted, and they frequently failed to maintain contact with the coverslip (Figure 2.3B). Quantitation of the coverslip contact area vs. time revealed that HS1-suppressed cells spread poorly and failed to undergo the sharp increase in contact area observed in control cells during the first 90 seconds (Figure 2.3C). To measure the irregular shape of HS1-suppressed cells, the variance in radial length was calculated. Control cells became round (low variance) by 120 seconds, while HS1-suppressed cells were irregularly shaped at all times (Figure 2.3D). These data show that TCR-stimulated actin

polymerization, ruffling and lamellipodial protrusion can occur in the absence of HS1, but HS1 is required for organizing and maintaining these structures.

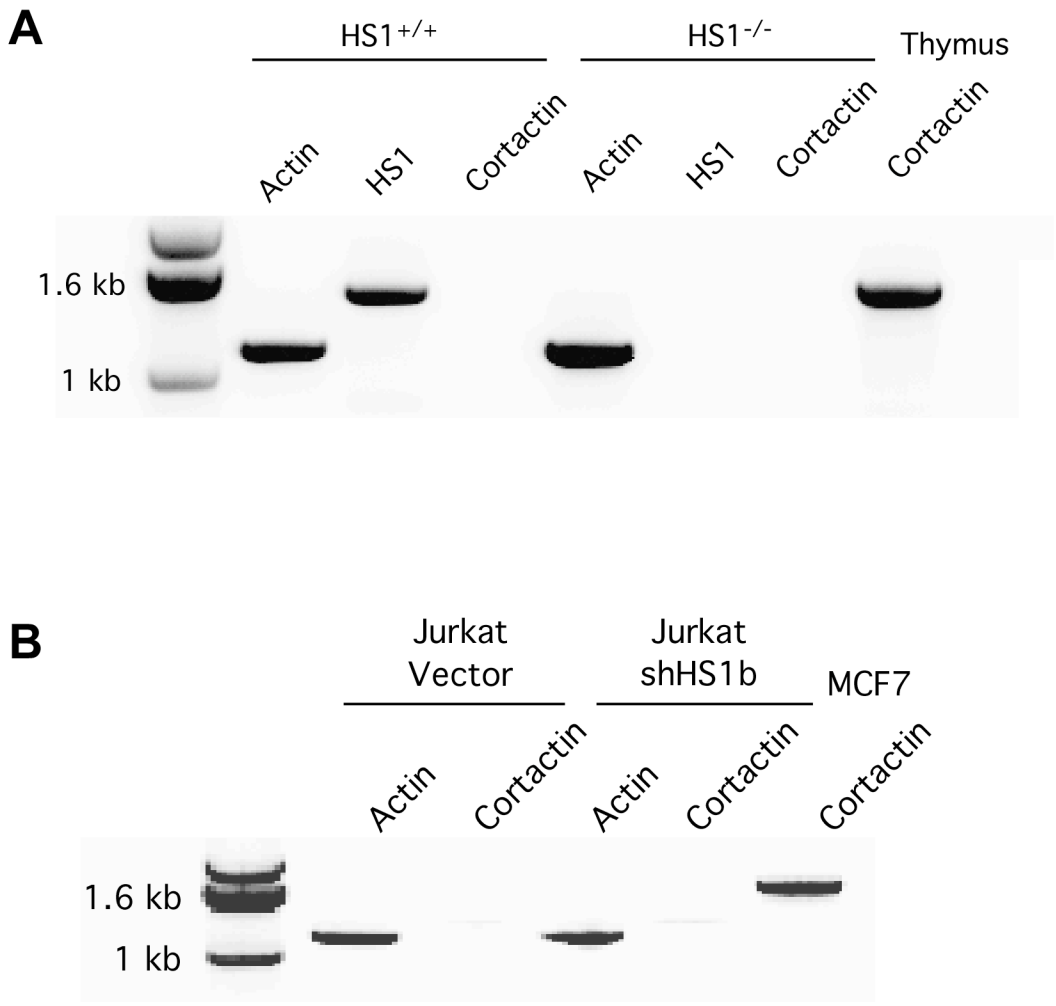

Figure 2.2: RT-PCR analysis of HS1 and cortactin expression.

RT-PCR analysis of HS1 and cortactin expression. Total RNA was extracted from secondary LN T cells from wild type or HS1-/- mice, or from total mouse thymus (A). Alternatively, RNA was extracted from Jurkat T cells transfected for 72 hours with empty suppression vector or shHS1b, or from MCF7 breast cancer cells (B). RT-PCR analysis was conducted for mouse (A) and human (B) HS1, cortactin and actin.

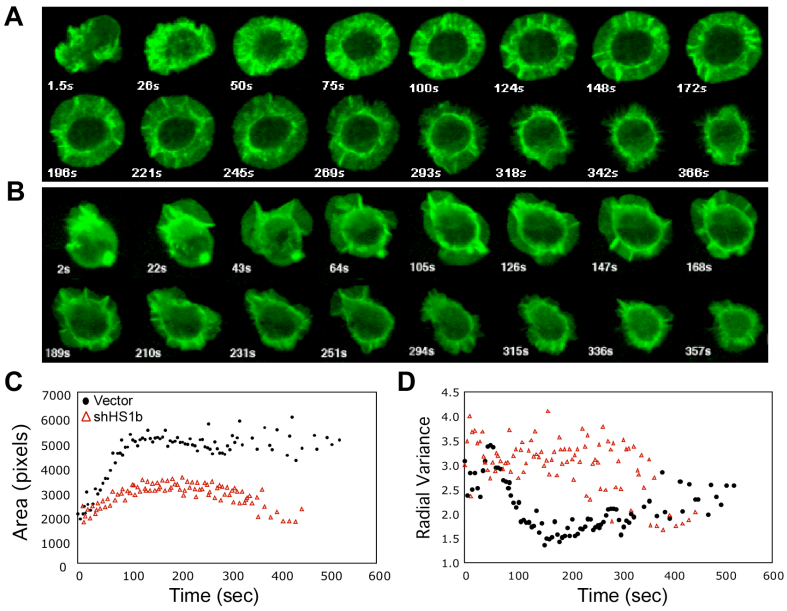

Figure 2.3: HS1-deficient cells exhibit abnormal actin dynamics in response to TCR engagement

Jurkat cells stably expressing GFP-actin were transfected with empty vector (A) or shHS1b (B). Actin dynamics were monitored by video confocal microscopy as the cells spread on anti-TCR-coated coverslips. Selected time-lapse image projections acquired at the indicated times after contact with the coverslip are shown**.** *C*, The contact area of each cell at each time point was determined, and the average calculated for each cell population at each 5s timepoint (17 cells for each condition). The difference in area was statistically significant (p=0.0002 at 135s). *D*, Irregularity of cell shape was assessed by measuring the radial variance of the cell outline for each cell at each time point, and calculating the average values for each time point. C and D, black circles, control transfectants; red triangles, HS1-suppressed cells.

Tyrosine phosphorylation of HS1 is required for actin remodeling

As reported previously (Hutchcroft et al., 1998; Takemoto et al., 1996; Yamanashi et al., 1993), TCR and CD28 ligation induced transient tyrosine phosphorylation of HS1 (Figure 2.4A). Stimulation with APCs had a similar effect, although phosphorylation was more sustained (Figure 3B). Phosphorylation requires both Lck and ZAP-70, since cells lacking these kinases failed to phosphorylate HS1 (Figure 2.4A).

To map the sites of HS1 phosphorylation, we conducted phosphopeptide mapping by mass spectrometry. We first tested recombinant HS1, which is phosphorylated *in vitro* by Syk at Y378 and Y397 (Brunati et al., 1999). HS1 yields a tryptic peptide of 4610 Da, which is shifted by the 80 Da mass of a single phosphate after phosphorylation by Syk (Figure 2.4C). This peptide includes both Y378 and Y360, but other peptides containing Y360 showed no evidence of phosphorylation (data not shown), indicating that phosphorylation occurs at Y378. This peptide, and another that includes Y397 (not shown) were confirmed as the major peptides phosphorylated by Syk *in vitro*. The phospho-peptide containing Y378 was also evident in HS1 immunoprecipitated from pervanadate-treated T cells, though it could not be identified with confidence in lysates from CD3-stimulated cells. A phosphopeptide containing only Y397 was clearly visible in material isolated from both pervanadate-treated and anti-CD3-activated cells. *In vitro* phosphorylation of HS1 at Y222 has been documented (Brunati et al., 1999; Ruzzene et al., 1996; Takemoto et al., 1996), and there is a report of Y198 phosphorylation in T cells (Salomon et al., 2003), however we did not detect phosphorylation at these sites. Thus,

we conclude that Y378 and Y397 are the principal sites for HS1 tyrosine phosphorylation in response to TCR stimulation. Consistent

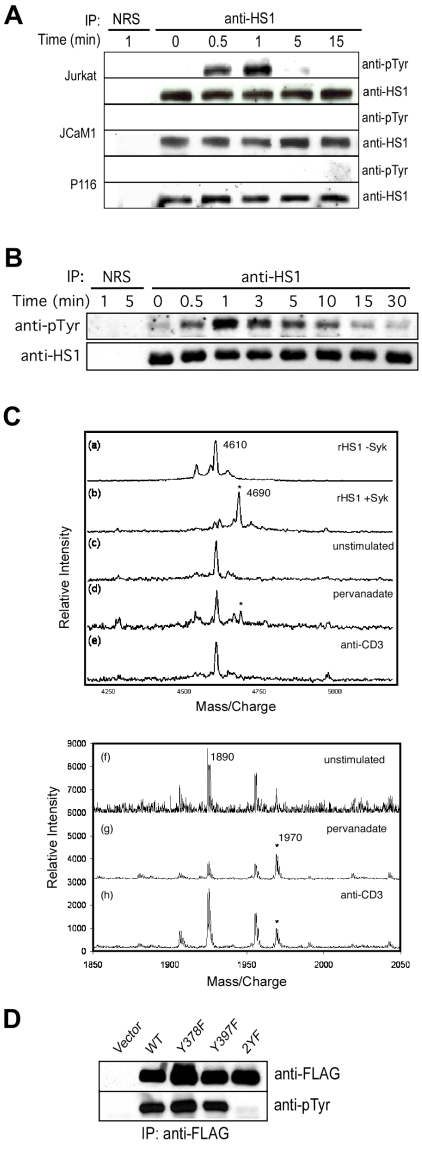
A
IP: NRS anti-HS1
Time (min) 1 0 0.5 1 5 15
Jurkat
JCaM1
P116
anti-pTyr
anti-HS1
B
IP: NRS anti-HS1
Time (min) 1 5 0 0.5 1 3 5 10 15 30
anti-pTyr
anti-HS1
C
(a) 4610 rHS1 -Syk
(b) 4690 rHS1 +Syk
(c) unstimulated
(d) pervanadate
(e) anti-CD3
Relative Intensity
Mass/Charge
(f) 1890 unstimulated
(g) 1970 pervanadate
(h) anti-CD3
Relative Intensity
Mass/Charge
D
Vector WT Y378F Y397F 2YF
anti-FLAG
anti-pTyr
IP: anti-FLAG

Figure 2.4: HS1 is phosphorylated at tyrosines 378 and 397 in stimulated T cells

A, Jurkat T cells or the Jurkat-derived cell lines JCaM1 (Lck-deficient) and P116 (ZAP-70-deficient) were stimulated with anti-TCR/anti-CD28. HS1 immunoprecipitates were immunoblotted for phospho-tyrosine. *B*, Jurkat T cells were unstimulated (time 0) or stimulated using fixed Raji B cells pulsed with SEE, and HS1 immunoprecipitates were immunoblotted for phospho-tyrosine. In A and B, rabbit IgG (NRS) was used as an immunoprecipitation control. *C*, Recombinant HS1 (rHS1, a and b) was incubated +/- Syk kinase. Alternatively, endogenous HS1 was immunoprecipitated from unstimulated (c and f), pervanadate-treated (d and g), or anti-CD3 stimulated (e and h) Jurkat cells. Following proteolysis, peptides were analyzed by Mass Spectroscopy. Top panel, tryptic peptides containing pY378 (asterisks), detected by MALDI-TOFF. Bottom panel, Glu-C peptides containing pY397 (asterisks) detected by v-MALDI ion trap. *D*, Jurkat T cells were transfected with FLAG-tagged WT HS1 or the Y378F, Y397F, and 2YF mutants. Cells were pervanadate-treated, the FLAG-tagged proteins immunoprecipitated, and immunoblotted with anti-phosphotyrosine.

with this, mutation of either individual residue (Y378F or Y397F) permitted tyrosine phosphorylation of the protein, whereas mutation of both sites (2YF) abrogated phosphorylation (Figure 2.4D).

To test the function of HS1 phosphorylation, T cells were transfected with YFP-tagged HS1 or HS1-2YF, and targeting to the IS was assessed. As shown in Figure 2.5A and B, YFP-HS1 colocalized with F-actin at the IS, but the 2YF mutant remained diffusely distributed. To express the HS1 mutant in cells lacking endogenous protein, we used a "suppression-re-expression" vector that encodes the HS1 shRNA targeting sequence together with shRNA-resistant wild type HS1 or HS1-2YF cDNA. This vector allows re-expression at near-endogenous levels (Figure 2.5C, right). Wild type HS1 restored actin polymerization at the IS, but the 2YF mutant did not (Figure 2.5C), indicating that phosphorylation of HS1 at Y378/Y397 is required for actin regulation at the IS.

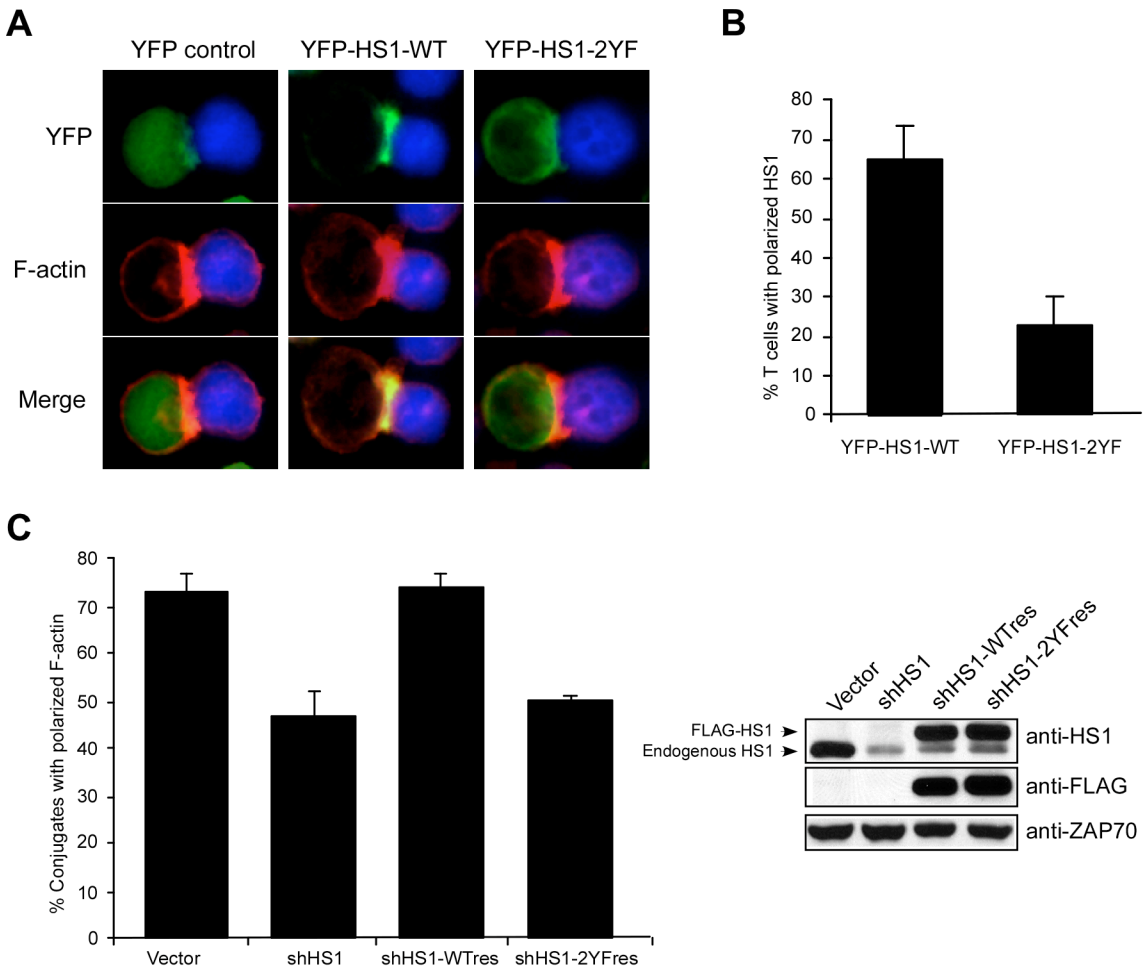

Figure 2.5: Phosphorylation at Y378/Y397 is required for HS1 localization and actin accumulation at the IS

A, Jurkat cells were transfected with YFP or YFP-tagged HS1 (WT or 2YF) and conjugated to SEE-pulsed Raji B cells (blue), and fixed conjugates were labeled with phalloidin. *B,* Conjugates formed as in A were scored for YFP-HS1 localization to the IS. *C,* Jurkat T cells transfected with the indicated constructs to allow suppression and re-expression of HS1 in the same cell. After 48h, cells were conjugated to SEE-pulsed NALM6 B cells, labeled with phalloidin, and conjugates containing GFP^+ T cells were scored for actin polarization. Immunoblot analysis shows HS1 suppression and re-expression. ZAP-70 serves as a loading control.

Tyrosine phosphorylation of HS1 mediates binding to key signaling molecules

SH2 domains from several proteins known to participate in T cell actin-regulatory pathways could bind phospho-HS1 in GST-pulldown assays (Figure 2.6A). In addition to Lck and Fyn, which are known to bind HS1 (Takemoto et al., 1996), both SH2 domains from the p85 subunit of PI3K, the second SH2 domain of PLCγ1 and the SH2 domains from all three Vav isoforms also interacted with phospho-HS1. To confirm these interactions *in vivo*, coimmunoprecipitation analysis was performed. Both PLCγ1 and Vav1 co-immunoprecipitated with HS1, with kinetics that mirrored HS1 tyrosine phosphorylation (Figure 2.6B). In contrast, Lck bound constitutively to HS1, with enhanced binding upon activation. This is consistent with previous work showing that HS1 binds both the SH3 and SH2 domains of Lck (Takemoto et al., 1996).

HS1 and Vav1 interact specifically and directly

Given the role of Vav1 in controlling actin responses, we focused on this interaction. As shown in Figure 2.6C, the GST-Vav1-SH2 fusion protein did not interact with overexpressed HS1-2YF from lysates of pervanadate-treated Jurkat cells; it bound weakly to the Y397F, and more strongly to Y378F HS1. An inactivating mutant of the GST-Vav1-SH2 (R696A) failed to bind HS1, confirming the specificity of binding. We conclude that the Vav1 SH2 domain can bind to both Y378 and Y397, but binds preferentially to Y397. To ask if HS1 binds Vav1 directly phosphorylated GST fusion peptides (p-GST) encompassing HS1 amino acids 361-436 were used in an *in vitro* pulldown of MBP-Vav1-SH2. The Vav1-SH2 bound directly to the WT, Y378F and

Y397F p-GST peptides, but not to the 2YF mutant (Figure 2.6D), mirroring the results from pervanadate-treated cells (Figure 2.6C). Direct interaction was also verified in a far-western blot (data not shown). These data show that the Vav1 SH2 domain binds directly to tyrosine phosphorylated HS1.

HS1 is required for maintaining Vav1 at the IS

Since Y378 and Y397 are required for HS1 targeting to the IS and for binding to Vav1, we reasoned that Vav1 might recruit HS1 to the IS. However, we found that HS1 recruitment was normal in T cells suppressed for Vav1 expression (Figure 2.6E). The converse experiment revealed a requirement for HS1 in localizing Vav1 to the IS (Figure 2.6F); HS1-suppressed cells initially recruit Vav1 to the IS, but Vav1 recruitment is abnormally short-lived. The kinetics of Vav1 loss closely resembles the kinetics of F-actin loss (Figure 2.1E). Importantly, this effect is specific to Vav1; WASp and WIP were recruited normally in HS1-suppressed cells (Figure 2.6G). These data show that one important function of HS1 is to stabilize Vav1 at the IS. As discussed below, this is likely to involve both direct and indirect interactions. Given the other actin-regulatory functions of HS1, it seems likely that HS1 and Vav1 function together to coordinate actin dynamics at the IS.

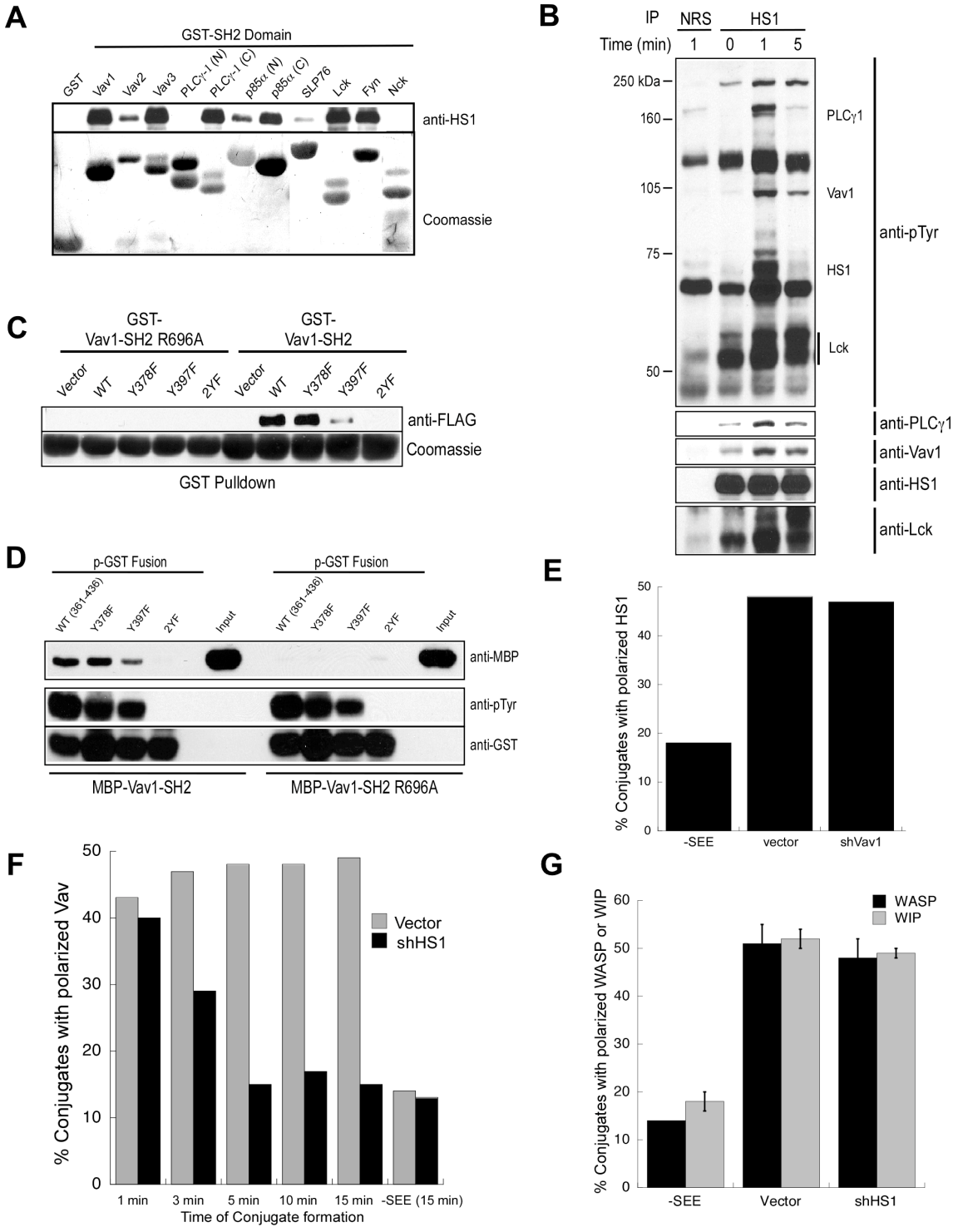
A
GST-SH2 Domain
GST
Vav1
Vav2
Vav3
PLCγ1 (N)
PLCγ1 (C)
p85α (N)
p85α (C)
SLP76
Lck
Fyn
Nck
anti-HS1
Coomassie
B
IP
NRS
HS1
Time (min)
1
0
1
5
250 kDa
160
105
75
50
PLCγ1
Vav1
HS1
Lck
anti-pTyr
anti-PLCγ1
anti-Vav1
anti-HS1
anti-Lck
C
GST-
Vav1-SH2 R696A
GST-
Vav1-SH2
Vector
WT
Y378F
Y397F
2YF
anti-FLAG
Coomassie
GST Pulldown
D
p-GST Fusion
WT (361-436)
Y378F
Y397F
2YF
Input
anti-MBP
anti-pTyr
anti-GST
MBP-Vav1-SH2
MBP-Vav1-SH2 R696A
E
% Conjugates with polarized HS1
-SEE
vector
shVav1
F
% Conjugates with polarized Vav
Vector
shHS1
1 min
3 min
5 min
10 min
15 min
-SEE (15 min)
Time of Conjugate formation
G
% Conjugates with polarized WASP or WIP
WASP
WIP
-SEE
Vector
shHS1

Figure 2.6: HS1 interacts with T cell signaling intermediates and functions to maintain Vav1 at the IS

A, Lysates from pervanadate-treated Jurkat cells were incubated with GSH-agarose-bound GST fusion proteins of various SH2 domains, and bound HS1 was detected by immunoblotting. Loading of fusion proteins was visualized with Coomassie. *B*, HS1 was immunoprecipitated from Jurkat cells following TCR/CD28 ligation, and interacting proteins were detected by immunoblotting. Rabbit IgG (NRS) was used as an immunoprecipitation control. *C*, Jurkat T cells were transfected with the indicated versions of FLAG-tagged HS1 and treated with pervanadate as in Figure 3D. Lysates were incubated with GSH-agarose-bound GST-Vav1-SH2 and the bound HS1 proteins were detected by immunoblotting with anti-FLAG. Loading of fusion proteins was visualized with Coomassie. *D*, GST fusion peptides encompassing AA361-436 of HS1 were created in a phosphorylated form (p-GST) as described in Experimental Procedures. HS1 p-GST fusion proteins corresponding to WT, Y378F, Y397F and 2YF were bound to GSH-agarose and tested for their ability to bind MBP-Vav1-SH2 in vitro. Phosphorylation of the p-GST fusions was visualized using anti-pTyr and bound MBP-Vav1 SH2 was detected with anti-MBP. 'Input' indicates the total amount of MBP-Vav-SH2 and Vav-SH2 R696A used per tube. In C and D, Vav1-SH2 domain containing an inactivating mutation (R696A) serves as control. *E*, Jurkat T cells were transfected with control or shVav1-GFP vectors, incubated for 15 min with NALM6 B cells +/- SEE, fixed, and labeled with anti-HS1. The frequency of conjugates containing GFP+ T cells exhibiting HS1 localization to the IS was scored. *F*, Jurkat cells were transfected with control or shHS1b-GFP vectors, fixed at the indicated times, and labeled with anti-Vav1. Conjugates containing GFP+ T cells were scored for Vav1 localization to the IS. *G*, Jurkat T cells were treated as in F, but labeled with anti-WASp or anti-WIP. Conjugates containing GFP+ T cells were scored for WASp or WIP localization to the IS. Data represent mean ±StDev from three experiments. Data in E and F are from one representative experiment.

HS1-mediated actin stabilization is required for sustained TCR signaling

Since T cells from HS1$^{-/-}$ mice have proliferative defects (Taniuchi et al., 1995), we asked if they exhibit defects in IL2 production. T cells from wild type and HS1$^{-/-}$ mice were cultured for 24 hours in the presence of SEB and wild type T-depleted splenocytes, and IL2 levels were measured. HS1$^{-/-}$ T cells produced significantly reduced levels of IL2 at all doses of SEB (Figure 2.7A). Analysis of IL2 reporter activity showed that HS1 suppression strongly inhibited activation of the *IL2* promoter, demonstrating that IL2 defects occur at the transcriptional level (Figure 2.7B).

The defects in HS1$^{-/-}$ T cells are not attributable to alterations in basal TCR expression levels, or in activation-induced downregulation of CD3 or upregulation of the activation markers CD25 and CD69 (Figure 2.7C). However, single cell analysis using ratiometric imaging showed a significant decrease in TCR-crosslinking-induced Ca^{2+} influx in HS1-suppressed cells and T cells from HS1$^{-/-}$ mice (Figure 2.8 A,B). Because Ca^{2+} signaling is required for NF-AT translocation, we assessed NF-AT-reporter activation. As shown in Figure 2.8C, NF-AT activity was significantly reduced in HS1-suppressed cells. Parallel analysis of NFκB also showed defects in this pathway (Figure 2.8D). Taken together, these results show that HS1 is dispensable for early TCR signaling events, but required for sustained signaling events leading to gene activation.

In the course of asking if HS1's role in controlling gene activation requires tyrosine phosphorylation of Y378/397, we found that IL2 reporter activity is exquisitely sensitive to the expression level of HS1. Overexpression of FLAG-tagged (or untagged)

HS1 inhibited *IL2* promoter activity in a dose-dependent fashion (Figure 2.8E and data not shown). Similar effects have been observed with other adapters, presumably because superstoichiometric expression leads to disruption of signaling complexes. In keeping with the idea that HS1 function involves tightly controlled protein levels, high-level overexpression of HS1 led to diminished levels of endogenous protein (Figure 2.8E, top). Although this overexpression effect made it impossible for us to conduct transcriptional analysis based upon re-expression of HS1 mutants in shRNA-suppressed cells, we found that the 2YF mutant lacks the inhibitory effects of the wild type molecule on IL2 promoter activity (Figure 2.8F). Thus, phosphorylation of HS1 is also important for activation of IL2 promoter activity.

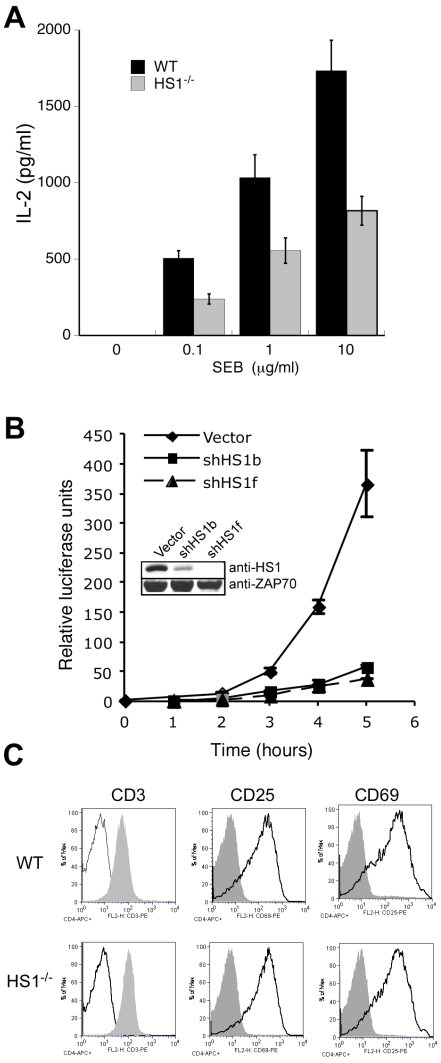
A
2000
1500
1000
500
0
IL-2 (pg/ml)
WT
HS1-/-
0
0.1
1
10
SEB (μg/ml)
B
450
400
350
300
250
200
150
100
50
0
Relative luciferase units
Vector
shHS1b
shHS1f
Vector
shHS1b
shHS1f
anti-HS1
anti-ZAP70
0
1
2
3
4
5
6
Time (hours)
C
CD3
CD25
CD69
WT
HS1-/-

Figure 2.7: HS1 is required for IL-2 production

A, T cells isolated from wt or HS1-/- mice were cultured for 24 h in the presence of WT T-depleted splenocytes and the indicated doses of SEB. Total IL-2 levels were measured by ELISA. Data are mean +/- StDev from replicate wells of one representative experiment. *B*, Jurkat T cells were co-transfected with IL2p-luc reporter and either vector control, shHS1b, or shHS1f plasmid. Cells were un-stimulated or stimulated with SEE-pulsed NALM6 B cells for 5h, and luciferase activity was measured hourly. Data represent mean ±StDev from triplicate samples. *C*, T cells isolated from WT and HS1-/- mice were unstimulated (shaded profiles) or stimulated for 24h with anti-CD3 and wt T-depleted splenocytes (open profiles). CD4+ T cells were analyzed for surface expression of CD3, CD25 and CD69 by flow cytometry.

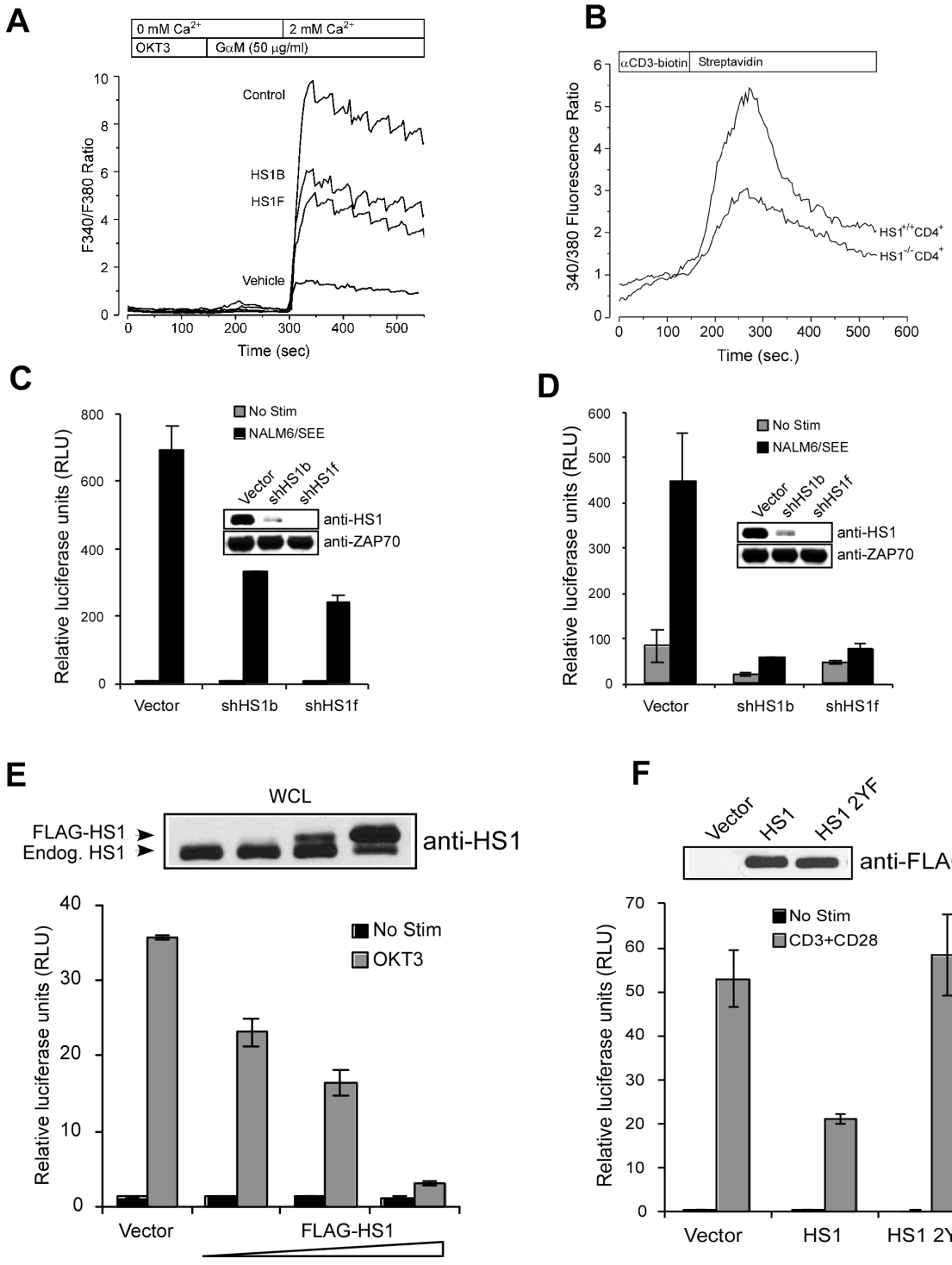
A
0 mM Ca2+
2 mM Ca2+
OKT3
GαM (50 μg/ml)
F340/F380 Ratio
Control
HS1B
HS1F
Vehicle
Time (sec)
B
αCD3-biotin
Streptavidin
340/380 Fluorescence Ratio
HS1+/+CD4+
HS1-/-CD4+
Time (sec.)
C
No Stim
NALM6/SEE
Vector
shHS1b
shHS1f
anti-HS1
anti-ZAP70
Relative luciferase units (RLU)
Vector
shHS1b
shHS1f
D
No Stim
NALM6/SEE
Vector
shHS1b
shHS1f
anti-HS1
anti-ZAP70
Relative luciferase units (RLU)
Vector
shHS1b
shHS1f
E
WCL
FLAG-HS1
Endog. HS1
anti-HS1
Relative luciferase units (RLU)
No Stim
OKT3
Vector
FLAG-HS1
F
Vector
HS1
HS1 2YF
anti-FLAG
Relative luciferase units (RLU)
No Stim
CD3+CD28
Vector
HS1
HS1 2YF

Figure 2.8: HS1 is required for sustained TCR-mediated signaling events leading to gene activation

A, Jurkat cells were transfected with control or shHS1 vectors. After 72h, TCR-induced Ca^{2+} signaling was examined using Fura-2 imaging. In Ca^{2+}-free bath, increases in Fura-2 ratio reflect Ca^{2+} release from intracellular stores. Extracellular Ca^{2+} entry via activated CRAC channels was then assessed by addition of extracellular Ca^{2+}. Each trace represents the average response of at least 100 cells. *B*, $CD4^{+}$ LN T cells from WT or $HS1^{-/-}$ mice were loaded with Fura-2, incubated with biotinylated anti-CD3 and activated by crosslinking with streptavidin. Analysis was as in A. Jurkat T cells were co-transfected with an NF-AT-luc *(C)* or NFkB-luc (D) reporter construct along with vector control, shHS1b, or shHS1f. Cells were un-stimulated or stimulated with SEE-pulsed NALM6 B cells and luciferase activity was measured. Jurkat cells were transfected with IL-2p-luc along with (E) vector control (40 μg) or an increasing concentration of FLAG-HS1 expression vector (10μg, 20μg, 40μg) or with (F) vector control, FLAG-HS1, or FLAG-HS1 2YF. Cells were un-stimulated or stimulated and luciferase activity was measured. The accompanying immunoblots show endogenous HS1 and FLAG-HS1 expression in whole cell lysates from each cell population. All bars represent mean ±StDev from triplicate samples.

HS1 is recruited to the periphery of spreading T cells

To more closely examine the localization of HS1 upon after TCR engagement, we generated a Jurkat cell clone stably expressing a variant of HS1 tagged at the N-terminus with the bright YFP variant Venus (Nagai et al., 2002). These cells expressed approximately a 1:1 ratio of tagged:endogenous HS1 (not shown). Cells spreading on anti-CD3-coated coverslips were observed by confocal microscopy over 3 minutes. HS1 was initially observed in discrete puncta and was enriched at the periphery throughout, in a pattern much like that observed for GFP-actin (Figure 2.9 and Supplementary Movies 2.8 and 2.9). These data suggest that HS1 is primarily localized to F-actin rich lamellipodia.

HS1 binding to F-actin is important for actin polymerization at the IS

A variant of HS1 lacking the third HTH repeat has been isolated from a patient with SLE (Sawabe et al., 2003). Since the HTH repeat region is involved in binding to F-actin (Hao et al., 2005), we reasoned that this disease-associated variant would provide an interesting opportunity to examine the role of actin binding in HS1 function. Hao *et al*. have reported that F-actin binding *in vitro* does not explicitly require the HTH repeat region and is instead more dependent on the CC domain, although F-actin binding in cells appears to be more complicated (Hao et al., 2005). To test the effect of loss of one HTH repeat on F-actin binding, recombinant GST-HS1^{EP6} or GST-HS1$^{HTH2.5}$ was incubated with increasing concentrations of F-actin and ultracentrifuged to separate F-actin-bound (pellet, P) from unbound (supernatant, s) protein. As shown in Figures 2.9A and 2.9B,

$HS1^{EP6}$ bound F-actin at all doses tested. In contrast, $HS1^{HTH2.5}$ bound less efficiently at all doses, and was fully unbound at the lowest dose of F-actin. We then asked if this defect in actin binding influences the ability of HS1 to promote Arp2/3 complex-dependent actin polymerization. Recombinant HS1 was incubated with Arp2/3 complex, and polymerization was initiated by the addition of actin. WASp VCA fragment was included as a positive control. $HS1^{EP6}$ promoted actin polymerization more efficiently than $HS1^{HTH2.5}$ (Figure 2.9C).

HS1 deficiency or mutation of Tyr 378 and Tyr 397 result in a failure to stabilize F-actin at the IS (Figure 2.1 and Figure 2.5, respectively). To determine if IS-associated F-actin structures depend on HS1, we expressed $HS1^{EP6}$ or $HS1^{HTH2.5}$ in T cells from $HS1^{-/-}$ mice, conjugated these cells to P815 cells bearing anti-CD3, and assessed actin responses. $HS1^{EP6}$ expression was able to rescue actin responses to WT levels, but $HS1^{HTH2.5}$ expression resulted only in a partial rescue of F-actin responses (Figures 2.11D and Figures 2.11E). These experiments need to be repeated to validate these results.

Discussion

In this study, we show that HS1 is required for the stabilization of F-actin filaments following TCR engagement, for maximal Ca^{2+} influx, and for NF-AT and NFkB-mediated gene transcription. In addition, we find that tyrosine phosphorylation is necessary for HS1 recruitment to the IS, and regulates its interaction with Lck, PLCγ1, and Vav1. These findings identify HS1 as an actin-regulatory adapter protein essential for T cell activation.

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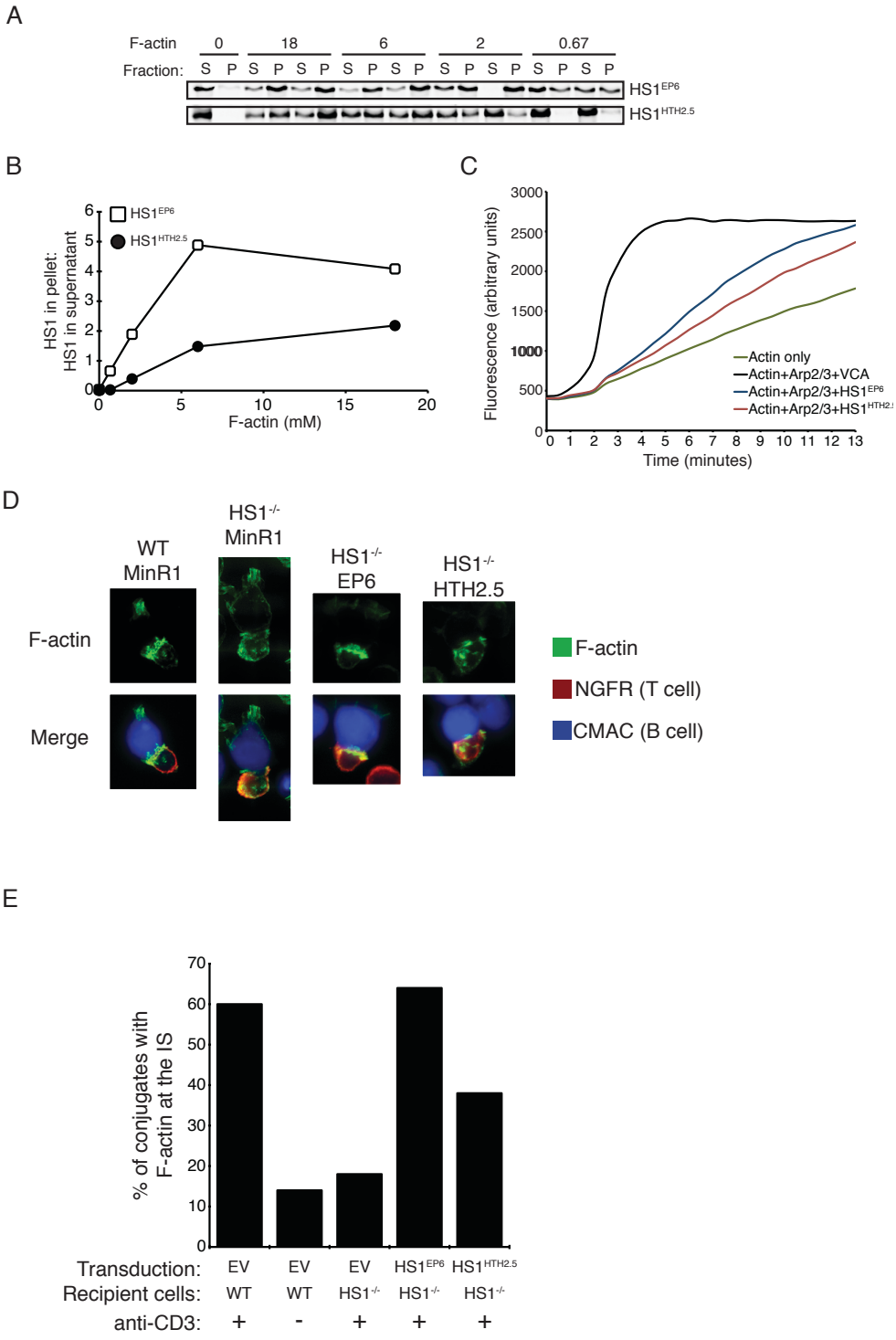
Figure 2.9: HS1 is recruited to the actin-rich periphery upon TCR engagement.

Top, Jurkat cells stably expressing Venus-HS1 were plated on anti-CD3-coated coverslips and imaged by confocal microscopy over the indicated time course after contact with the coverslip (seconds). *Bottom*, The 20 second time point is expanded, and HS1-rich clusters are marked with red arrows. This time-lapse sequence corresponds to supplemental movie 2.8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F-actin 0 18 6 2 0.67
Fraction: S P S P S P S P S P S P S P S P S P
HS1EP6
HS1HTH2.5
B
HS1EP6
HS1HTH2.5
HS1 in pellet:
HS1 in supernatant
F-actin (mM)
C
Fluorescence (arbitrary units)
Time (minutes)
Actin only
Actin+Arp2/3+VCA
Actin+Arp2/3+HS1EP6
Actin+Arp2/3+HS1HTH2.5
D
WT MinR1
HS1-/- MinR1
HS1-/- EP6
HS1-/- HTH2.5
F-actin
Merge
F-actin
NGFR (T cell)
CMAC (B cell)
E
% of conjugates with F-actin at the IS
Transduction: EV EV EV HS1EP6 HS1HTH2.5
Recipient cells: WT WT HS1-/- HS1-/- HS1-/-
anti-CD3: + - + + +

Figure 2.10: HS1-F-actin binding is important for actin responses.

A, GST-HS1^{EP6} or GST-HS1$^{HTH2.5}$ was incubated with the indicated concentrations of F-actin and fractionated by ultracentrifugation. F-actin bound (pellet, P) and unbound (supernatant, S) fractions were analyzed by immunoblotting for GST. *B*, Quantitation of the blot shown in *A*. Data are expressed as the ratio of HS1 in the supernatant to HS1 in the pellet. *C*, GST-HS1^{EP6} or GST-HS1$^{HTH2.5}$ was incubated with Arp2/3 and G-actin containing 20% pyrene-labeled monomers. Polymerization was measured over the indicated time course. WASp VCA fragment was included as a positive control. *D*, WT or HS1$^{-/-}$ T cells (red) were transduced with the indicated constructs and conjugated to P815 cells (blue) and stained as in Figure 2.1F. *E*, Conjugates in *D* were analyzed for the conjugates of cells with F-actin at the IS.

HS1-deficient T cells show unique defects in actin polymerization at the IS; actin-rich structures are formed initially, but are unstable and erratic. This phenotype is distinct from that of cells lacking WASp or WAVE2, both of which also bind Arp2/3 complex. Cells lacking WASp spread essentially normally, while cells lacking WAVE2 fail to spread altogether (Nolz et al., 2006). The phenotype of HS1-deficient T cells resembles that of cortactin-deficient fibroblasts, which display disorganized lamellipodial formation (Bryce et al., 2005; Kempiak et al., 2005). These phenotypes are consistent with the idea that by binding F-actin as well as Arp2/3 complex, these proteins inhibit debranching and stabilize cortical actin (Uruno et al., 2003b). An unresolved question is whether HS1 also acts via direct interactions with WASp and WIP, as reported for cortactin (Kempiak et al., 2005; Kinley et al., 2003; Tehrani et al., 2007; Uruno et al., 2003a; Weaver et al., 2002; Weaver et al., 2001). Though we can readily detect binding of HS1 to the WIP/WASp complex in vitro, we have so far failed to verify interaction by co-immunoprecipitation from T cell lysates. This may reflect weak interactions that are disrupted upon T cell lysis. Nonetheless, we show that HS1 is not required for recruitment of WASp or WIP to the IS. Indeed, their continued presence likely contributes to the residual actin dynamics in HS1-deficient cells.

We mapped the major sites of HS1 tyrosine phosphorylation in activated T cells to amino acids 378 and 397, and our data suggest that ZAP-70 is responsible for phosphorylating these sites. Y378 and Y397 are critical for several aspects of HS1 function. Mutation of these residues leads to defective HS1 targeting to the IS, abrogates binding to Vav1, and perturbs actin responses. Finally, these residues are required for

overexpression-induced inhibition of IL2 promoter activation. Phosphorylation-dependent membrane targeting may be a general feature of HS1 function; in B cells, tyrosine phosphorylation of HS1 mediates its recruitment to lipid rafts (Hao et al., 2004). Although we have failed to detect HS1 in lipid raft fractions from T cell (not shown), we have found that the Tec family kinase Itk binds to these tyrosines, and mediates HS1 recruitment to the IS. These studies are described in Chapter 3. While it is possible that the functional defects observed in T cells expressing HS1 2YF are solely attributable to the aberrant localization of this mutant, it seems more likely that phosphorylation regulates multiple aspects of HS1 function. In addition to mediating binding to other signaling molecules (discussed below), phosphorylation may induce conformational changes that influence HS1 function, as already shown for cortactin (Huang et al., 1997a; Martinez-Quiles et al., 2004).

One clear role of HS1 phosphorylation is to mediate binding to SH2-domain-containing proteins, including Lck, Vav1, and PLCγ1. The functional significance of Vav1 binding is underscored by the finding that HS1-suppressed T cells progressively lose Vav1 from the IS. Interestingly, the loss of Vav1 from the IS in HS1-suppressed cells parallels the loss of F-actin at this site. This, and finding that the kinetics Vav1/F-actin loss differ from those of HS1 phosphorylation, suggest that HS1 stabilizes Vav1 at the IS via a complex mechanism. Since Vav1 recruitment to the IS dependents on interactions with SLP-76 and Itk (Dombroski et al., 2005; Zeng et al., 2003), we propose that SLP-76, Itk and HS1 coordinately recruit Vav1 to the IS at early time points. Once this complex is localized to the IS, Vav1 initiates actin polymerization through activation

of Cdc42 and Rac. HS1 participates in forming and/or stabilizing F-actin at later times, and this feeds back to stabilize Vav1 interactions with the SLP-76 complex. Stabilization could occur through direct interactions or via the F-actin scaffold. The fact that HS1 binds directly to Vav1 suggests that these important actin regulatory proteins function coordinately. It will be interesting to ask if this interaction modifies the activity of either protein.

Phosphorylation also mediates HS1 interaction with PLCγ1, and studies further probing this interaction are described in Chapter 3. Regulated interactions with PLCγ1 and the p85a subunit of PI-3K may also play a role in HS1-mediated actin regulation via effects on inositol phospholipids.

High-resolution live-cell microscopy has shown that signaling molecules coalesce within signaling microclusters at sites of TCR engagement (Bunnell et al., 2002; Yokosuka et al., 2005). We have found that HS1 is transiently recruited to puncta reminiscent of these microclusters, but primarily redistributes to the actin-rich periphery, where active signaling predominates. Phosphorylation likely occurs at both sites. These data support the idea that HS1 primarily functions to link active signaling complexes to the actin cytoskeleton, although further investigation is necessary. In Chapter 3, this concept is explored further.

We showed here, using a disease-associated variant of HS1, that efficient F-actin binding is important for HS1-mediated Arp2/3 complex-dependent actin polymerization and for actin responses at the IS. From the data presented here, it is not possible to judge whether the observed partial defect in actin polymerization *in vitro* represents a true

defect in Arp2/3 complex activation, or whether it is due to premature disassembly of polymerized actin throughout the course of the assay. It will be interesting to examine the effect of this actin binding mutation on signaling to the *IL2* promoter.

Initial analysis of $HS1^{-/-}$ mice demonstrated defects in T cell proliferation and negative selection (Taniuchi et al., 1995) but did not address the molecular basis of these defects. We show that $HS1^{-/-}$ T cells have defective actin and Ca^{2+} responses, as well as defective IL2 production associated with defects in activation of NF-AT and NFkB transcriptional elements. It remains to be determined how alterations in HS1 function result in the observed changes in IL2 promoter activation. Perturbations in signaling through Vav1 and/or PLCγ1 may be involved, since both proteins are required for activation of NF-AT and NFkB (Cao et al., 2002; Costello et al., 1999; Dolmetsch et al., 1998; Irvin et al., 2000). The role of HS1 in regulating Ca^{2+} signaling is further explored in Chapter 3.

Chapter 3: Recruitment of HS1 to the immunological synapse by Itk regulates actin responses and PLCγ1 microcluster dynamics[3]

Summary

Productive T cell activation requires efficient reorganization of the actin cytoskeleton. We showed previously that the actin regulatory protein hematopoietic lineage cell-specific protein 1 (HS1) is required for the stabilization of F-actin and Vav1 at the immunological synapse (IS) and for efficient calcium responses. The Tec family kinase Itk regulates similar aspects of T cell activation, suggesting that these proteins act in the same pathway. Using video microscopy, we show that T cells lacking Itk or HS1 exhibited similar defects in actin responses, extending unstable lamellipodial protrusions upon TCR stimulation. HS1 and Itk could be co-immunoprecipitated from T cell lysates, and GST-pulldown studies showed that Itk's SH2 domain binds directly to two phosphotyrosines in HS1. In the absence of Itk, or in T cells overexpressing an Itk SH2-domain mutant, HS1 failed to localize to the IS, indicating that Itk serves to recruit HS1 to sites of TCR engagement. Since Itk is required for PLCγ1 phosphorylation and

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[3] This chapter is a modified form of paper entitled, "Hematopoietic lineage cell-specific protein 1 is recruited to the immunological synapse by IL-2-inducible T cell kinase and regulates phospholipase Cγ1 microcluster dynamics during T cell spreading," published in the December 1 issue of the Journal of Immunology, Volume 183, pages 7352-7361, with the following author list: Esteban Carrizosa, Timothy S. Gomez, Christine M. Labno, Deborah A. Klos Dehring, Xiaohong Liu, Bruce D. Freedman, Daniel D. Billadeau, and Janis K. Burkhardt.

calcium store release, we examined the calcium signaling pathway in HS1$^{-/-}$ T cells in greater detail. In response to TCR engagement, T cells lacking HS1 exhibited abnormal calcium store release but normal TCR-dependent PLCγ1 phosphorylation. However, these cells exhibited defective cytoskeletal association of PLCγ1 and altered formation of PLCγ1 microclusters. We conclude that Itk-dependent recruitment of HS1 mediates actin stabilization at the IS, and directs the spatial organization of PLCγ1 signaling complexes.

Introduction

Effective T cell activation requires reorganization of the actin cytoskeleton. The interaction between a T cell and an antigen presenting cell (APC) bearing cognate antigen results in the activation of multiple actin regulatory proteins, which promote the polymerization of actin filaments at the immunological synapse (IS) (Burkhardt et al., 2008). T cells lacking these molecules or treated with pharmacological agents that disrupt actin dynamics exhibit functional defects in their responses to antigen. Commonly, diminished calcium (Ca^{2+}) mobilization responses are observed, resulting in poor IL2 production and proliferation. Although the molecular mechanisms by which actin dynamics influence T cell signaling are poorly understood, visualization of high-order signaling complexes using live-cell imaging has begun to yield some insights. Proper actin dynamics are required for the formation of signaling microclusters at the cell periphery and for their movement toward the center, while the maintenance of centralized microclusters is largely independent of the actin cytoskeleton (Campi et al., 2005; Varma et al., 2006). Peripheral and central microclusters also appear to be functionally distinct

with respect to their role in signaling. Though details differ depending on experimental conditions (Cemerski et al., 2008), newly formed, peripheral, microclusters are thought to be the predominant sites for early phosphorylation events, while central microclusters are usually associated with signal down-regulation (Seminario and Bunnell, 2008). Thus, actin dynamics are required to assemble active signaling complexes, and to drive the mechanism that ultimately extinguishes signaling.

HS1 is an actin regulatory protein expressed throughout the hematopoeitic system (Kitamura et al., 1989). Like its more widely expressed homologue, cortactin, HS1 binds to the Arp2/3 complex and actin filaments, and is thought to stabilize branched actin filaments (Uruno et al., 2003b; Weaver et al., 2001). We have shown that T cells lacking HS1 can generate actin-rich lamellipodial protrusions in response to T cell receptor (TCR) engagement, but these protrusions are disordered and rapidly collapse (Gomez et al., 2006). Consistent with this observation, accumulation of F-actin at the IS is abnormally short-lived. HS1-deficient T cells also exhibit defects in Ca^{2+} signaling leading to diminished IL2 production. The regulation of HS1 activity is not fully understood. TCR engagement leads to rapid phosphorylation of HS1 at tyrosines 378 and 397. This is required for recruitment of HS1 to the IS, and for its binding to numerous key signaling proteins, including the Rac-1/Cdc42 guanine nucleotide exchange factor Vav1. While T cells lacking HS1 exhibit defects in the maintenance of Vav1 at the IS, Vav1 is dispensable for recruitment of HS1 to the IS (Gomez et al., 2006). How HS1 is recruited to the IS is unknown.

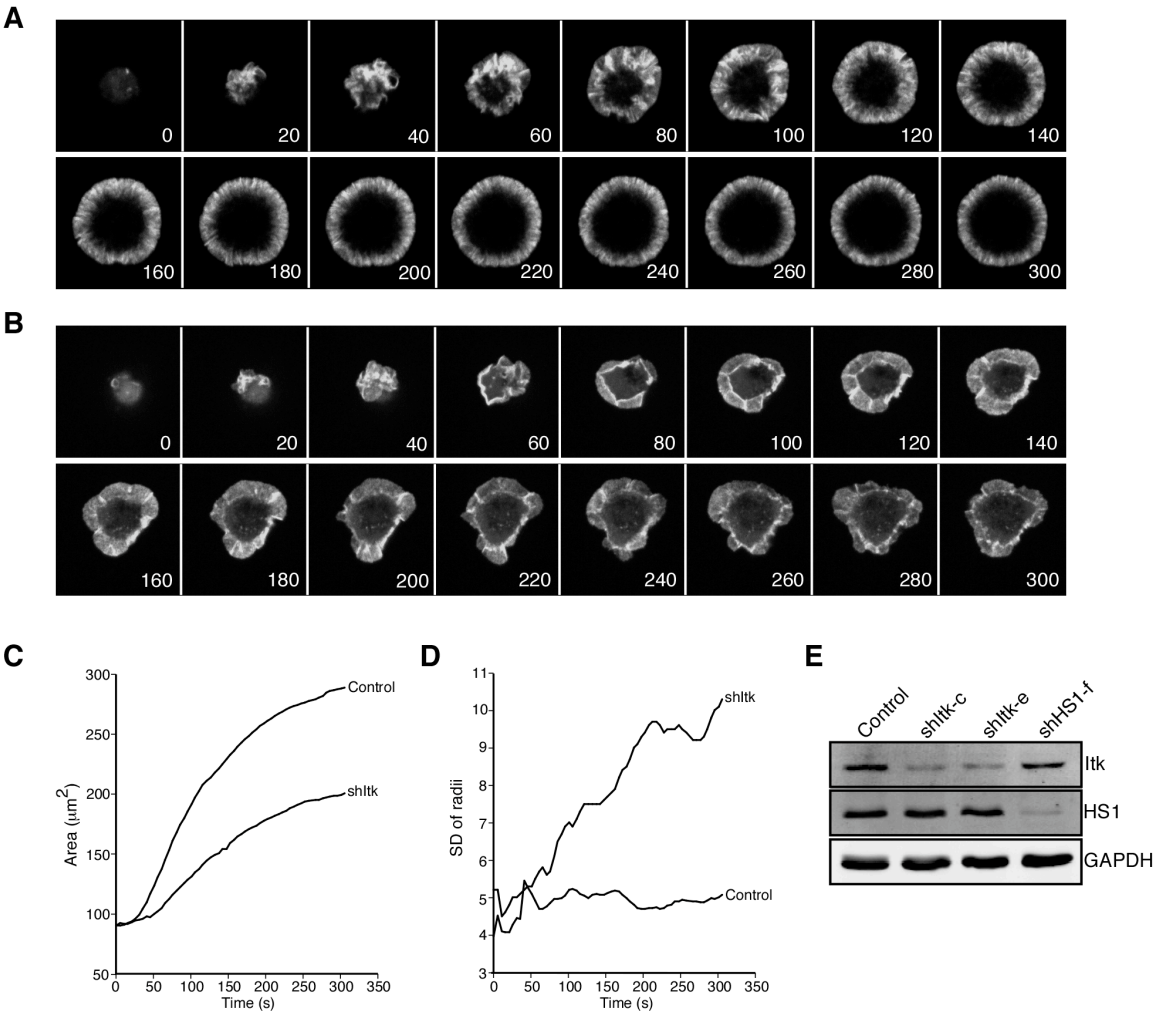
TCR ligation-induced activation of actin-regulatory proteins, including HS1, depends on signaling through tyrosine kinases and adapter proteins (Reviewed in (Burkhardt et al., 2008)). Although these molecules do not directly influence the actin cytoskeleton, cells lacking them exhibit severe defects in actin dynamics. We and others have shown that T cells lacking the Tec family kinase Itk fail to polymerize actin properly in response to TCR ligation (Dombroski et al., 2005; Grasis et al., 2003; Labno et al., 2003), and fail to recruit Vav1 to the IS (Dombroski et al., 2005). Interestingly, the actin-regulatory role of Itk is independent of kinase activity, and depends instead on adapter functions carried out by its SH2 domain (Dombroski et al., 2005; Grasis et al., 2003).

To begin to understand how the interactions between actin regulatory proteins and upstream regulators lead to productive T cell activation, we examined the possibility of an interaction between HS1 and Itk. Using live cell imaging, we now show that cells lacking Itk exhibit similar defects in actin dynamics to those lacking HS1. The SH2 domain of Itk binds to phosphotyrosines in HS1, and this interaction is required for the recruitment of HS1 to the IS. Recruitment of HS1 to the IS then promotes productive Ca^{2+} signaling by modulating the formation of PLCγ1 microclusters. Taken together, these studies show that Itk-HS1 interactions play an important role in controlling actin-driven dynamics of TCR signaling microclusters leading to productive T cell activation.

Results

Itk-deficient T cells show unstable lamellipodial protrusions

Previous studies have shown that Itk is required for proper actin responses in a manner dependent on its SH2 domain, but not its kinase activity (Dombroski et al., 2005; Grasis et al., 2003; Labno et al., 2003). To better understand how Itk regulates T cell actin responses, we generated Itk-deficient T cells using RNA interference (RNAi), (Figure 3.1E), and visualized actin dynamics in these cells during spreading on coverslips coated with anti-CD3. As previously shown (Bunnell et al., 2001), control Jurkat T cells expressing GFP-actin extended stable actin-rich lamellipodia and exhibited retrograde actin flow from the periphery of the cell toward the center (Figure 3.1A and Supp. Videos 3.1 and 3.2). Itk-deficient T cells also extended actin-rich lamellipodia, but these structures were disordered and retracted frequently (Figure 3.1B and Supp. Videos 3.3 and 3.4). The overall spreading area of Itk-suppressed cells was less than that of wild-type cells (Figure 3.1C). Furthermore, whereas control cells maintained a regular, round profile, Itk-suppressed cells were irregularly shaped. This difference is illustrated by an increased radial variance in the Itk-suppressed cells relative to controls (Figure 3.1D). The phenotype of Itk-deficient T cells in this assay is strikingly similar to that of T cells lacking HS1, an actin-binding protein that is thought to stabilize branched actin filaments generated in response to TCR engagement. Like Itk-suppressed cells, HS1-suppressed cells exhibit diminished spreading and irregular and unstable lamellipodial protrusions (Chapter 2, (Gomez et al., 2006), and Supp. Videos 3.5 and 3.6).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C
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shItk
Area (μm2)
Time (s)
D
shItk
Control
SD of radii
Time (s)
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Control
shItk-c
shItk-e
shHS1-f
Itk
HS1
GAPDH

Figure 3.1: Itk-deficient T cells exhibit unstable lamellipodial protrusions.

A, Jurkat T cells stably expressing GFP-actin were transfected with empty suppression vector. After 72 hours, cells were plated on coverslips coated with anti-CD3 and imaged by confocal microscopy for the indicated times (seconds). Selected images from one time-lapse series are shown; these correspond to Supplemental video 1. *B*, Jurkat cells stably expressing GFP-actin were transfected with Itk suppression vector and analyzed as in *A*. Selected images from one time-lapse series are shown; these correspond to Supplemental video 3. *C*, The contact area of each cell at each time point was determined and the average was calculated for each cell population at each 5 s time point (control=46 cells, shItk=49 cells). *D*, Irregularity of cell shape was assessed by measuring the radial variance for each cell at each time point and calculating the average values. *E*, Western blot analysis showing suppression of Itk or HS1 in GFP-actin Jurkat cells transfected with the indicated suppression vectors.

The SH2 domain of Itk mediates binding to HS1

To test the possibility that HS1 is an effector for Itk in the T cell actin regulatory pathway, we assessed interaction between these two proteins by co-immunoprecipitation. As shown in Figure 3.2, Itk was found in association with HS1 in both activated and resting mouse $CD4^+$ T cell blasts. Similar results were obtained using Jurkat T cells; moreover, HS1 was present in Itk immunoprecipitated (data not shown).

To map the site(s) of interaction between the two proteins, we used a GST pulldown approach. Domain maps of HS1 and Itk are shown in Figure 3.3A. Itk has SH3 and SH2 domains that could bind to proline-rich sequences or phosphotyrosines in HS1, respectively. Conversely, the SH3 domain of HS1 could bind to the proline-rich region of Itk. To test the role of the Itk SH3 and SH2 domains, we generated a panel of GST-fusion constructs encoding the wild-type SH3-SH2 domain fragment of Itk, or with inactivating point mutations in the SH3 domain, the SH2 domain or both. These fragments were used to probe Jurkat T cell lysates for binding of HS1. As shown in Figure 3.3B, HS1 bound to the wild-type Itk fragment in an activation-dependent manner. This binding was unaffected by mutation of the Itk SH3 domain, but was abolished upon mutation of the SH2 domain, consistent with binding mediated by SH2 domain-phosphotyrosine interactions. In some experiments, we detected modest HS1 binding to the wild-type construct in the absence of pervanadate treatment (data not shown). However, this was also abolished upon mutation of the SH2 domain, suggesting that this interaction is attributable to basal tyrosine phosphorylation of HS1. To ask if the SH3

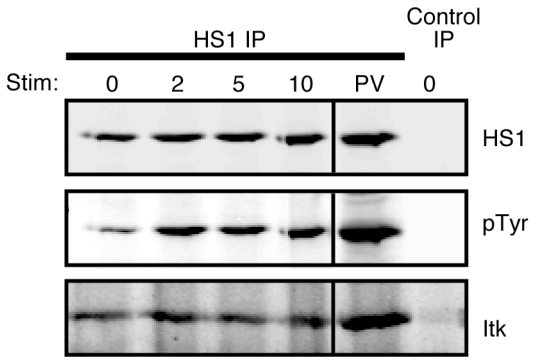
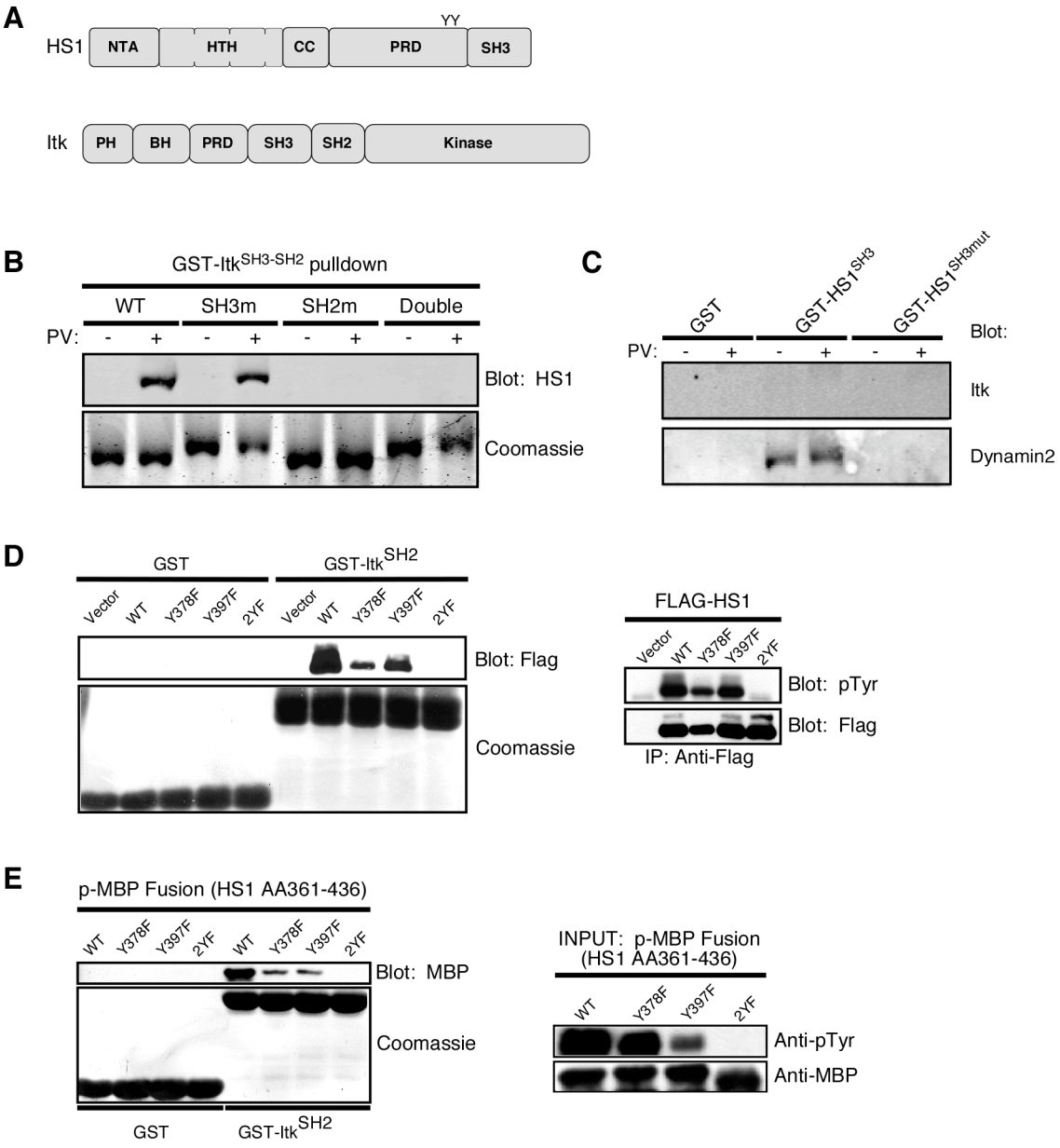


Figure 3.2: HS1 and Itk interact in T cells

HS1 and Itk interact in T cells. $CD4^+$ T cell blasts were stimulated with anti-CD3 for the indicated times (minutes), treated with pervanadate (PV), or left untreated. HS1 was immunoprecipitated from lysates, and immunoprecipitates were analyzed by western blotting for HS1, phosphorylated HS1 (by blotting for total phosphotyrosine, pTyr), or Itk. Control immunoprecipitation was performed using pre-immune rabbit serum.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WT
SH3m
SH2m
Double
PV:
Blot: HS1
Coomassie
C
GST
GST-HS1 SH3
GST-HS1 SH3mut
PV:
Blot:
Itk
Dynamin2
D
GST
GST-Itk SH2
Vector
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Y378F
Y397F
2YF
Blot: Flag
Coomassie
FLAG-HS1
Blot: pTyr
Blot: Flag
IP: Anti-Flag
E
p-MBP Fusion (HS1 AA361-436)
Blot: MBP
Coomassie
GST
GST-Itk SH2
INPUT: p-MBP Fusion
(HS1 AA361-436)
Anti-pTyr
Anti-MBP

Figure 3.3: The SH2 domain of Itk binds directly to phosphorylated HS1.

A, Domain maps of HS1 and Itk. *B,* A panel of recombinant GST fusion proteins comprised of the wild-type SH3-SH2 fragment of Itk and inactivating point mutants in either domain were coupled to glutathione resin and incubated with lysates from Jurkat cells that had been treated with pervanadate (PV) or left untreated. The pulldown reactions were washed and analyzed by western blotting for HS1 (top) or Coomassie staining to monitor the GST-Itk proteins (bottom). *C,* The wild-type GST-HS1-SH3 domain or an inactivating point mutant were coupled to glutathione resin and incubated with lysates from Jurkat cells that had been treated with pervanadate (PV) or left untreated. The pulldown reactions were washed and analyzed by western blotting for Itk or Dynamin 2. *D,* Jurkat cells were transfected with expression vectors encoding wild-type FLAG-tagged HS1 or the indicated point mutants. These cells were treated with pervanadate, lysed, and probed with GST-Itk SH2 domain. Left, bound proteins were analyzed by western blotting for FLAG or Coomassie staining to monitor the GST-Itk proteins. Right, FLAG immunoprecipitates were probed for phosphotyrosine and FLAG to monitor phosphorylation status of HS1 mutants. *E,* Purified recombinant GST-Itk SH2 domain was incubated with recombinant HS1 phosphopeptide fused to myelin basic protein (MBP). Left, bound proteins were analyzed by western blotting and Coomassie staining. Right, purified MBP fusion peptides were analyzed by western blotting for phosphotyrosine and MBP.

domain of HS1 provides an additional means of interaction with Itk, we conducted reciprocal pulldown experiments using the HS1 SH3 domain. However, Itk binding to the HS1 SH3 domain could not be detected, even under conditions where interactions with other binding partners could be verified (Figure 3.3C). We conclude that HS1 binding to Itk depends predominantly on interactions between the SH2 domain of Itk and tyrosine phosphorylation sites in HS1.

Phosphorylation at Y378 and Y397 of HS1 mediates binding to many SH2 domains (Chapter 2 and (Gomez et al., 2006)), making these sites the likely points of interaction between HS1 and Itk. To test this hypothesis, we transfected Jurkat cells with vectors expressing wild-type FLAG-tagged HS1, or HS1 point mutants at one or both tyrosines. Transfected T cells were treated with pervanadate and lysates were probed for binding of FLAG-HS1 to the SH2 domain of Itk. As shown in Figure 3.3D, wild-type HS1 bound efficiently. Mutation of either tyrosine substantially reduced binding, and mutation of both sites abolished the interaction altogether. This shows that both tyrosines 378 and 397 contribute to the interaction with Itk.

To determine if HS1 and Itk interact directly, we asked whether the recombinant GST-Itk SH2 domain could bind to phosphorylated recombinant HS1 fusion protein in the absence of other components of the T cell signaling machinery. As Figure 3.3E shows, HS1 and Itk interacted directly, and efficient binding required both Y378 and Y397. We conclude that HS1 and Itk interact in T cells and that this interaction involves direct binding between the SH2 domain of Itk and tyrosines 378 and 397 of HS1. Since we have shown previously that these sites on HS1 are phosphorylated transiently upon

TCR engagement ((Gomez et al., 2006) and Chapter 2), direct interaction between HS1 and Itk is predicted to be transient, and the observed constitutive association between these proteins (Figure 3.2) is likely to involve additional interactions with mutual binding partners.

Itk is required for HS1 recruitment to the IS

We have shown previously that phosphorylation of HS1 at Y378 and Y397 is required for its recruitment to the IS (Chapter 2 and (Gomez et al., 2006)). Since these sites mediate direct binding to the SH2 domain of Itk, we asked if Itk is required for recruitment of HS1 to the IS. As shown in Figure 3.4A, control Jurkat cells conjugated to superantigen-pulsed B cells recruited HS1 to the IS, but this recruitment was impaired in cells transfected with an Itk suppression vector. In contrast, T cells lacking HS1 showed no defect in recruitment of Itk to the IS (Figure 3.4B), indicating that the requirement for recruitment is unidirectional. To quantitate the requirement for Itk in recruiting HS1 to the IS, conjugates were prepared using control and Itk-suppressed Jurkat cells, and the frequency of conjugates showing a bright band of HS1 at the IS was scored. As shown in Figure 3.4C, Itk suppression diminished HS1 recruitment to frequencies similar to those observed in the absence of superantigen. Using a similar approach, we asked what portions of Itk are required to mediate HS1 recruitment. Jurkat T cells were transfected with wild-type Itk, the kinase-dead (K390R) mutant, or the SH2 domain (R265A) mutant, and HS1 recruitment to the IS was assessed. As shown in Figure 3.4D**,** overexpression of wild-type or kinase dead Itk had no effect on HS1 localization, but overexpression of the

Itk SH2 domain mutant efficiently inhibited HS1 recruitment to the IS. The requirements for HS1 recruitment with respect to Itk parallel those for actin recruitment (Dombroski et al., 2005). We conclude that Itk, through its SH2 domain, recruits HS1 to the IS to promote TCR-induced actin responses.

HS1 is required for efficient Ca^{2+} store release

In addition to its role in regulating actin responses, Itk is a key signaling intermediate in TCR signaling-induced Ca^{2+} responses (Liu et al., 1998; Schaeffer et al., 1999). Itk is required for phosphorylation of PLCγ1, leading to Ins(1,4,5)P_3 production and release of Ca^{2+} from intracellular stores. HS1 is also required for normal T cell Ca^{2+} responses (Gomez et al., 2006), but the level at which HS1 functions in this pathway has not been addressed. To test this, $HS1^{-/-}$ T cells were stimulated with anti-CD3 in the absence of extracellular Ca^{2+} to visualize ER store release, and then exposed to extracellular Ca^{2+} to permit Ca^{2+} influx via CRAC channels. $HS1^{-/-}$ T cells showed a significant blunting of Ca^{2+} responses (Figure 3.5A). Expansion of the Y-axis to better show the response in the absence of extracellular Ca^{2+} (Figure 3.5A, right panel) reveals that HS1-deficient T cells fail to show significant release of Ca^{2+} from ER stores. To confirm this finding, T cells were treated with the SERCA pump inhibitor thapsigargin to pharmacologically induce store depletion, and Ca^{2+} responses were assessed. As shown in Figure 3.5B, treatment with thapsigargin rescued the Ca^{2+} defect in HS1-deficient T cells, indicating that HS1, like Itk, is required for Ca^{2+} store release.

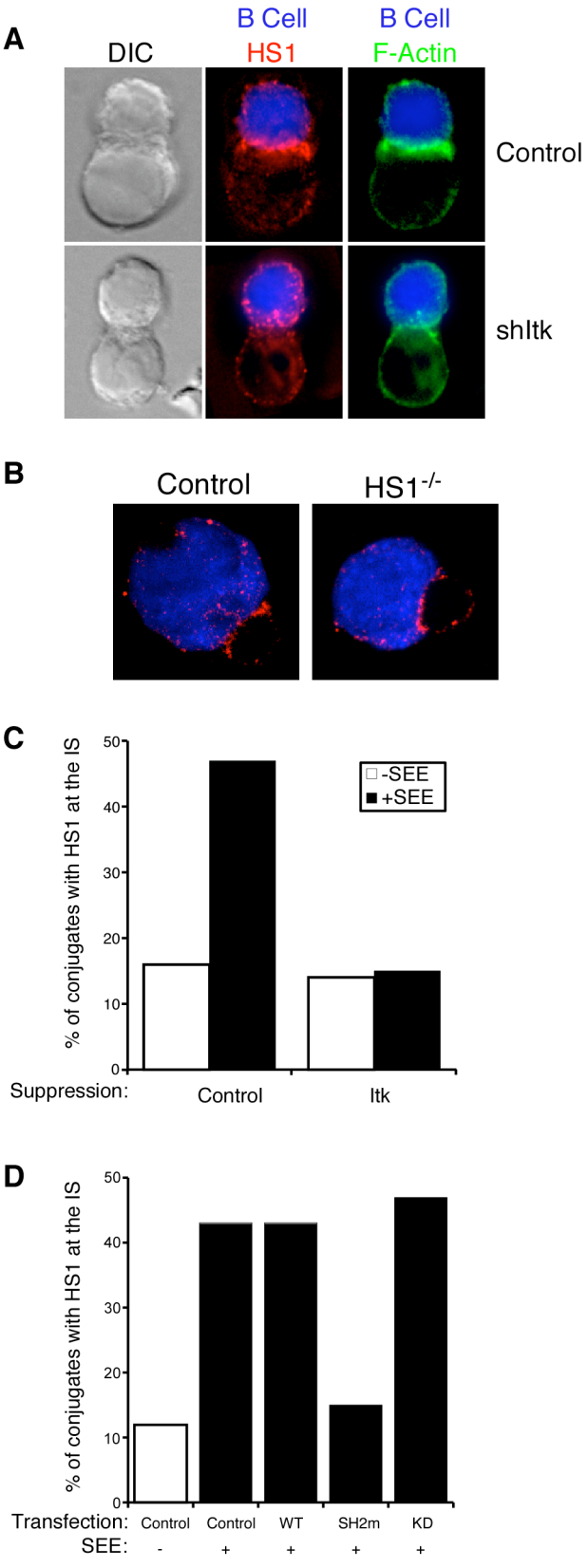

A
DIC
B Cell
HS1
B Cell
F-Actin
Control
shItk
B
Control
HS1-/-
C
% of conjugates with HS1 at the IS
50
40
30
20
10
0
-SEE
+SEE
Suppression:
Control
Itk
D
% of conjugates with HS1 at the IS
50
40
30
20
10
0
Transfection:
Control
Control
WT
SH2m
KD
SEE:
-
+
+
+
+

Figure 3.4: The SH2 domain of Itk is required for recruitment of HS1 to the IS.

A, Jurkat cells transfected with Itk suppression or control vectors were conjugated to CMAC-stained, SEE-loaded Raji B cells (blue) for 15 minutes. Conjugates were fixed and stained for HS1 (red) or F-actin (green). *B,* $CD4^+$ T cells from $HS1^{+/-}$ or $HS1^{-/-}$ AND TCR transgenic mice were conjugated to CMAC-stained, MCC peptide-loaded CH27 B cells for 3 minutes and stained for Itk (red). *C,* Itk-suppressed or control Jurkat T cells conjugated to Raji B cells were scored for recruitment of HS1 to the cell-cell contact site. *D,* Jurkat cells were transfected with expression vectors for wild-type, SH2 domain mutant (SH2m), or kinase-dead (KD) Itk. Conjugates were prepared and analyzed as in C. Data in C and D represent averages of at least 50 conjugates from one representative experiment.

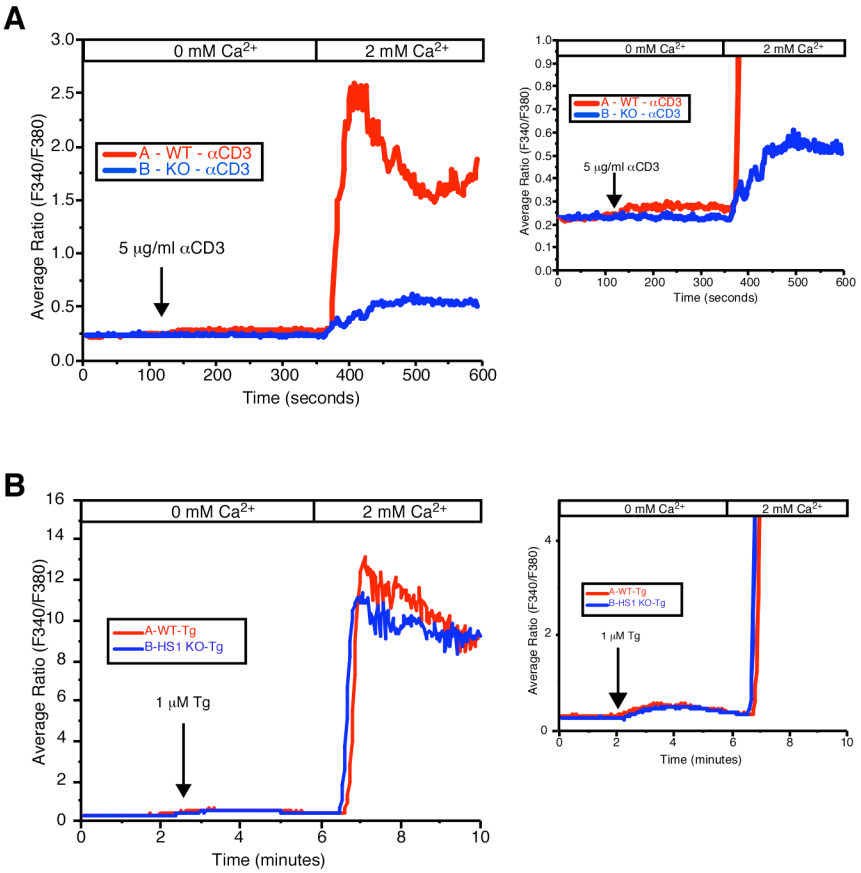


Figure 3.5: HS1 is required for release of Ca^{2+} from intracellular stores.

HS1 is required for release of Ca^{2+} from intracellular stores. *A,* $CD4^{+}$ T cells from $HS1^{+/+}$ or $HS1^{-/-}$ mice were loaded with the Fura-2AM and plated on poly-L-lysine-coated coverslips. Immediately before TCR stimulation, the cells were superfused with Ca^{2+}-free bath solution. At the indicated time, cells were stimulated by the addition of anti-CD3 (500.A2) and analyzed by fluorescence microscopy to visualize a rise in cytoplasmic Ca^{2+} indicative of release from ER stores. Extracellular Ca^{2+} was then added to visualize Ca^{2+} influx. *Right*, an expansion of the Y-axis to visualize release from ER stores. *B,* Cells prepared as in A were treated with 1 μM thapsigargin (Tg) and imaged as in *A*.

Previous studies have demonstrated that at least one Itk binding partner, SLP-76, can activate Itk kinase activity toward PLCγ1 (Bogin et al., 2007). To ask if HS1 plays a similar role, T cells from wild-type and $HS1^{-/-}$ mice were stimulated with anti-CD3 for various times and the phosphorylation of PLCγ1 was analyzed by western blotting using site-specific antibodies. As shown in Figure 3.6A, phosphorylation of PLCγ1 at tyrosine 783 proceeded normally in the absence of HS1. Phosphorylation at tyrosine 775 was also intact (data not shown). Thus, we conclude that HS1 is not required for phosphorylation-dependent activation of PLCγ1. PLCγ1 activation is characterized not only by phosphorylation, but also by localization to the IS, in association with other components of the LAT signalosome (Braiman et al., 2006). We therefore analyzed PLCγ1 localization in wild-type and $HS1^{-/-}$ AND TCR transgenic T cells conjugated to MCC-pulsed CH27 B cells. As shown Figure 3.6B, PLCγ1 localized efficiently to the IS at early time points in both control and HS1-deficient T cells. Time course analysis showed that wild-type cells were able to maintain PLCγ1 at the IS for 20 minutes, whereas $HS1^{-/-}$ cells showed significant loss of IS-associated PLCγ1 by 10 minutes. This mirrors the defects we have previously observed for F-actin and Vav1 (Gomez et al., 2006). Nonetheless, given the rapid kinetics of Ca^{2+} store release relative to the defects we observed in PLCγ1 localization, the Ca^{2+} signaling defects we observe in HS1-deficient T cells cannot be explained by a gross failure to recruit PLCγ1 to the IS.

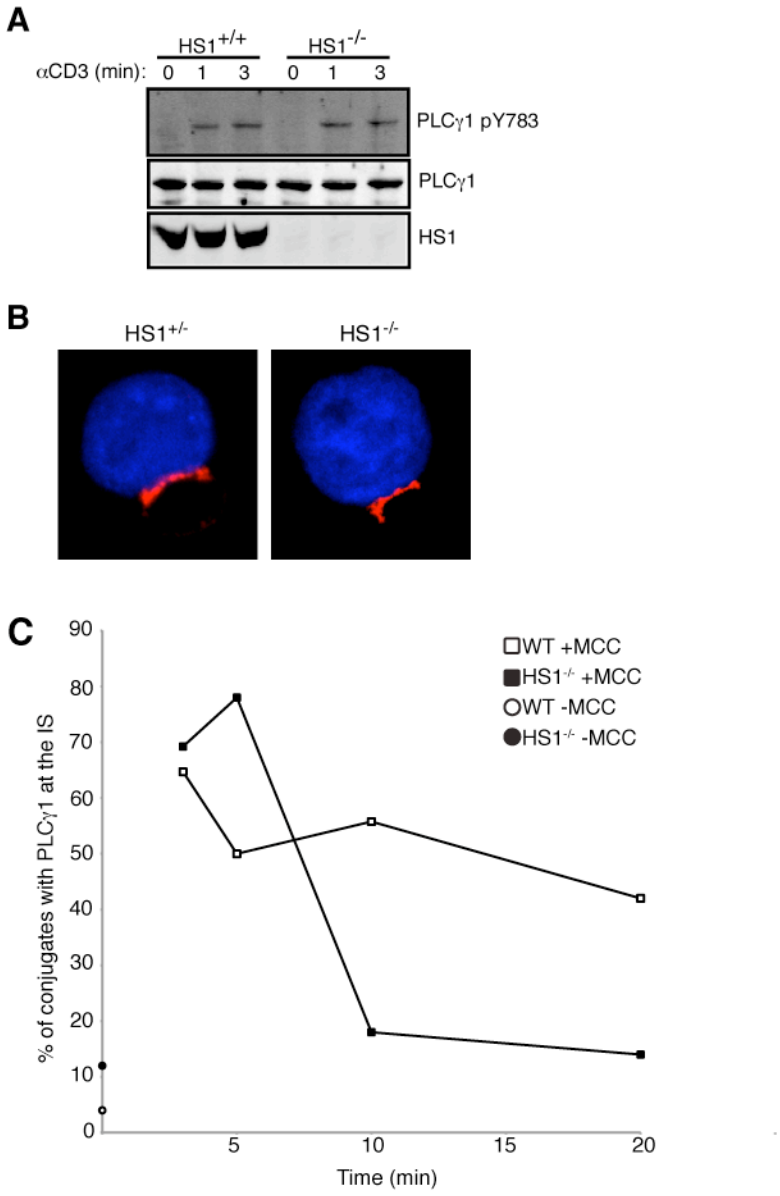

A
HS1+/+
HS1-/-
αCD3 (min): 0 1 3 0 1 3
PLCγ1 pY783
PLCγ1
HS1
B
HS1+/-
HS1-/-
C
% of conjugates with PLCγ1 at the IS
90
80
70
60
50
40
30
20
10
0
5
10
15
20
Time (min)
WT +MCC
HS1-/- +MCC
WT -MCC
HS1-/- -MCC

Figure 3.6: HS1 is dispensable for PLCγ1 phosphorylation and early recruitment to the IS.

A, T cells from $HS1^{+/+}$ or $HS1^{-/-}$ mice were stimulated as indicated and lysates were analyzed by western blotting for PLCγ1 phosphorylated at Y783, total PLCγ1, and HS1. *B*, $CD4^{+}$ T cells from $HS1^{+/-}$ or $HS1^{-/-}$ AND TCR transgenic mice were conjugated to CMAC-stained, MCC peptide-loaded CH27 B cells (blue) and stained for PLCγ1 (red). *C,* The fraction of conjugates with PLCγ1 at the indicated times was determined.

The finding that sustained PLCγ1 localization to the IS depends on HS1 led us to examine earlier actin-driven aspects of PLCγ1 signaling in greater detail. To ask if HS1 functions to modulate PLCγ1 association with the cytoskeleton, we prepared insoluble actin-rich fractions from wild-type or $HS1^{-/-}$ T cells, and assessed the association of total and phospho-PLCγ1 with these fractions by western blotting. In wild-type T cells, a fraction of PLCγ1 became associated with the cytoskeleton within 1 minute of TCR engagement (Figure 3.7A). PLCγ1 continued to accumulate in the cytoskeletal fraction over the next five minutes (Figure 3.7B). Importantly, this pool was enriched in phospho-PLCγ1 such that the vast majority of phospho-PLCγ1 was cytoskeletally associated (Figures 3.7A and 3.7C). In contrast, little or no activation-induced cytoskeletal association of PLCγ1 was observed in lysates from $HS1^{-/-}$ T cells. Although the experiment shown in Figure 7A shows increased phospho-PLCγ1 in the cytoskeletal fraction at 3 minutes in $HS1^{-/-}$ T cells, this result was not reproducible. The significance of the insoluble pool of PLCγ1 remains to be defined; however, given that the phosphorylated pool of the molecule is thought to represent the active pool, it seems likely that cytoskeletal association is intimately linked with PLCγ1 function. Nonetheless, these results support the idea that HS1 affects PLCγ1 organization during early phases of TCR signaling.

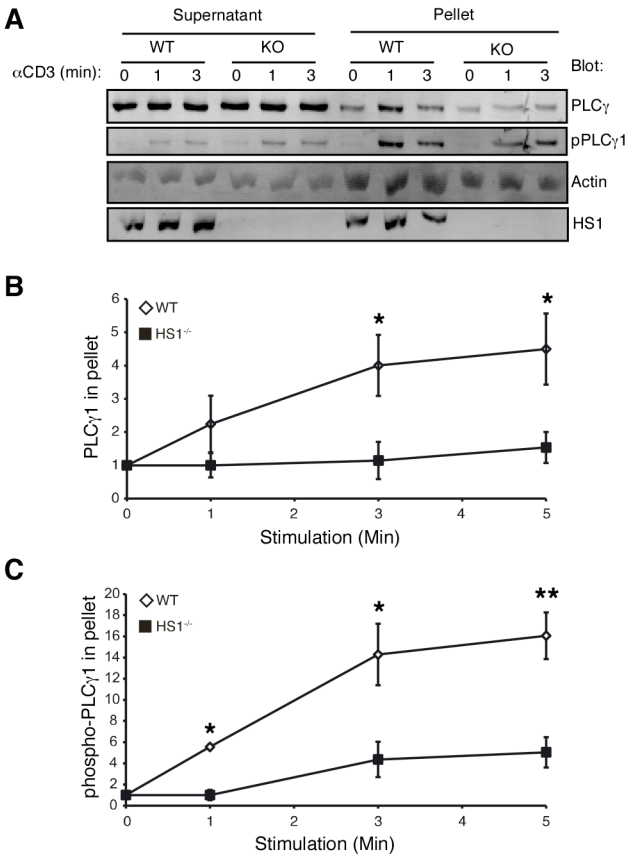


Figure 3.7: HS1 is required for PLCγ1 cytoskeletal association

A, T cells from $HS1^{+/+}$ or $HS1^{-/-}$ mice were stimulated as indicated, lysed in cytoskeletal stabilizing lysis buffer, and separated into cytosol-enriched supernatant and F-actin rich pellet fractions. Fractions were analyzed by western blotting as indicated. pPLCγ1 was detected using an antibody specific for PLCγ1 phosphorylated at Y783. *B*, The amount of PLCγ1 in the pellet fractions at each time point was quantified and normalized to the amount of PLCγ1 in the pellet in unstimulated cells. Data represent averages from four independent experiments +/- SEM. *, $p<0.05$. *C*, The amount of PLCγ1 phosphorylated at Y783 in the pellet fractions at each time point was quantified and normalized to the amount of PLCγ1 phosphorylated at Y783.

To observe directly the effects of HS1 on PLCγ1 dynamics, we analyzed the formation of signaling microclusters in Jurkat cells expressing PLCγ1-eYFP during spreading on anti-CD3 coated coverslips. In control cells, as reported previously (Braiman et al., 2006), PLCγ1 assembled into microclusters at the T cell-coverslip interface. As spreading progressed, new microclusters were formed within actin-rich lamellipodial protrusions, near the edge of the spreading cell (Figure 3.8A). HS1-suppressed cells also formed microclusters, but these clusters were often disorganized and formed large aggregates (Figure 3.8B). The formation of microclusters at the cell periphery was also impaired, presumably because HS1-deficient cells do not spread normally. To assess this phenotype, individual microclusters were identified in video sequences, and pseudocolored in order of appearance. As shown in Figure 3.8C, in control cells, microclusters were formed at distinct sites in a concentric fashion, with newer microclusters in the periphery. In contrast, HS1-deficient T cells exhibited formation of new microclusters that overlapped with older ones, resulting in aggregates of mixed age (Figure 3.8D). We assessed the size of these mixed aggregates in each cell at 90 seconds after contact with the coverslip. As shown in Figure 3.8E, suppression of HS1 resulted in a two-fold increase in average microcluster size. This increase in average size was attributable to an abnormally disparate size distribution. As shown in Figure 3.8F, the vast majority of microclusters in control cells were less than 0.5 μm in diameter. HS1-deficient cells also showed numerous small microclusters, but nearly half of the microclusters in these cells exceeded 0.5 μm, with some as large as 6 μm. Since

peripheral microclusters are key sites for early TCR signaling events (Campi et al., 2005; Varma et al., 2006; Yokosuka et al., 2005), the defect in Ca^{2+} responses in HS1-deficient T cells may reflect a role for HS1 in controlling PLCγ1 signaling at the level of microcluster dynamics.

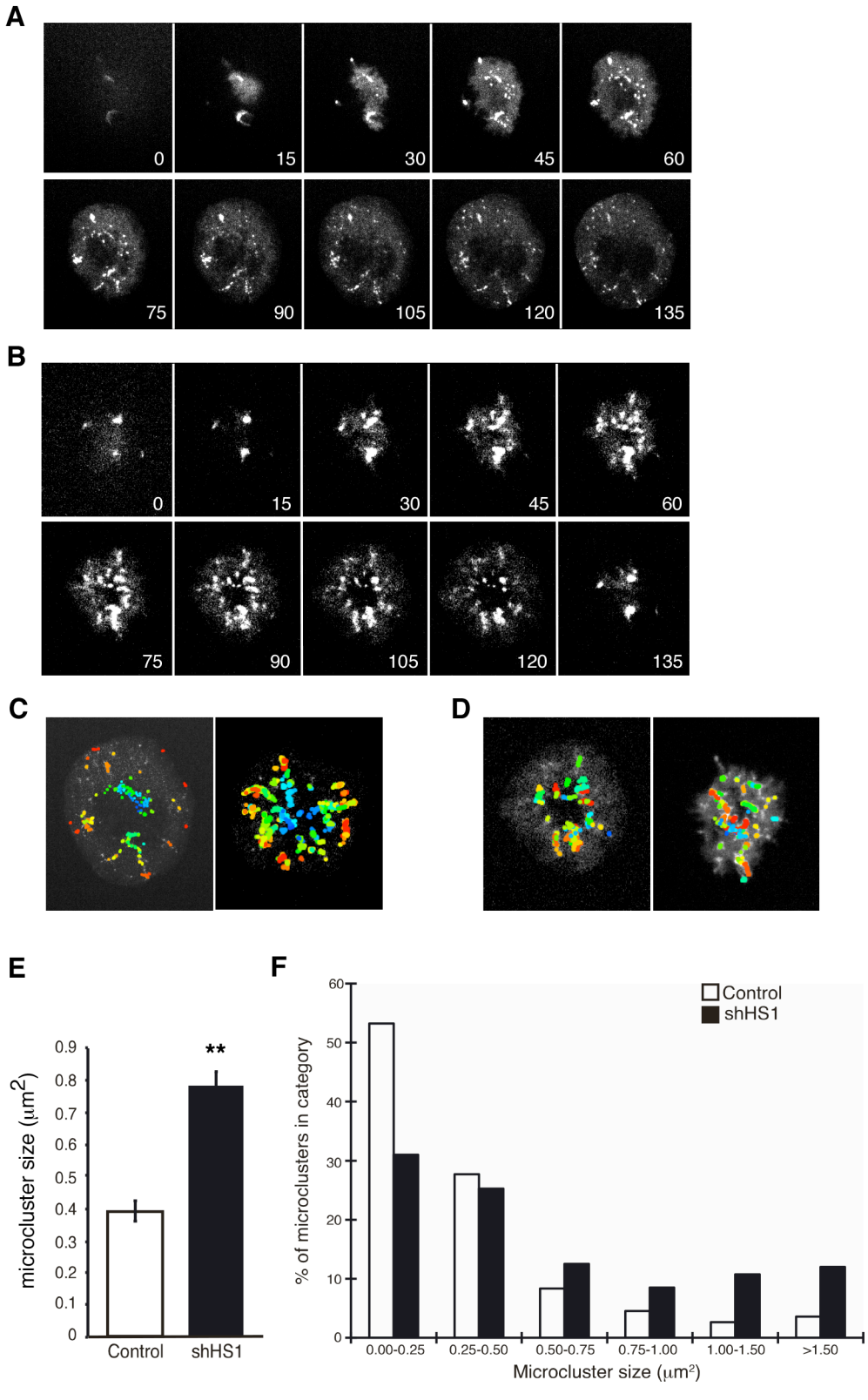

A
0
15
30
45
60
75
90
105
120
135
B
0
15
30
45
60
75
90
105
120
135
C
D
E
microcluster size (μm2)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
**
Control
shHS1
F
% of microclusters in category
60
50
40
30
20
10
0
Control
shHS1
0.00-0.25
0.25-0.50
0.50-0.75
0.75-1.00
1.00-1.50
>1.50
Microcluster size (μm²)

Figure 3.8: HS1 regulates PLC1 microcluster dynamics

A, Jurkat cells stably expressing PLCγ1-eYFP were transfected with empty vector, plated on coverslips coated with anti-CD3, and imaged by confocal microscopy. Selected images from one time-lapse series are shown; these correspond to Supplemental video 7. *B*, Jurkat cells stably expressing PLCγ1-eYFP were transfected with HS1 suppression vector and analyzed as in *A*. Selected images from one time-lapse series are shown; these correspond to Supplemental video 9. *C and D*, PLCγ1 microclusters in empty vector *(C)* or HS1-suppressed *(D)* T cells were identified and color coded by order of appearance, with newer microclusters colored orange and red, and older microclusters colored blue and green. Left hand panels show cells from the sequences shown shown in *A* and *B*. Right hand panels show additional examples. *E*, The area of individual microclusters was determined at 90 seconds after the initiation of contact with the coverslip. At least 20 cells were analyzed per condition, yielding approximately 400 microclusters each. Data represent mean +/- SEM. **, $p<0.0001$. *F*, The areas of individual microclusters from *E* were grouped into the indicated bins. The percent of total microclusters falling into each size category is shown.

Discussion

Regulation of actin dynamics in T cells involves coordination of multiple actin regulatory proteins by upstream kinases including Lck, ZAP70, and c-Abl (Huang et al., 2008; Lowin-Kropf et al., 1998; Morgan et al., 2001). Itk has also been demonstrated to play a role in regulating T cell actin responses, but this function is independent of its kinase activity and instead requires its SH2 domain (Dombroski et al., 2005; Donnadieu et al., 2001; Grasis et al., 2003; Labno et al., 2003). We show here that one role of the SH2 domain of Itk is to recruit HS1 to the IS. Video analysis shows that T cells lacking Itk or HS1 have similar defects in actin reorganization downstream of TCR engagement. In contrast to T cells lacking WAVE2 or Arp2/3 complex components, which fail to extend actin-rich lamellipodial protrusions (Gomez et al., 2007; Nolz et al., 2006), T cells deficient for HS1 or Itk can extend these structures, but they are unstable and retract frequently. This phenotype is consistent with *in vitro* studies showing that HS1 functions to stabilize existing branched actin filaments (Uruno et al., 2003b; Weaver et al., 2001). In addition to stabilizing actin filaments directly, HS1, like Itk, has been implicated in regulating the localization of Vav1 at the IS (Gomez et al., 2006). Localization of Vav1 to the IS requires an intact Itk SH2 domain (Dombroski et al., 2005). Since Vav1 binds directly to HS1, this suggests that HS1 links Vav1 to Itk during T cell activation. It is important to point out, however, that these proteins also interact with other signalosome components including LAT, SLP-76, and PLCγ1, such that loss of any one of these molecules disrupts interactions among the others and perturbs T cell actin responses (Bunnell et al., 2006). Finally, there is evidence that the actin scaffolds generated by

these signaling molecules function to stabilize newly formed signaling complexes (Campi et al., 2005), thereby generating a positive feedback loop to facilitate T cell activation.

We have mapped binding of the Itk SH2 domain to phosphotyrosines 378 and 397 of HS1. These residues are phosphorylated upon TCR engagement in a ZAP70-dependent manner and are required for recruitment of HS1 to the IS and for its functions in actin remodeling and signaling to the *Il2* promoter (Gomez et al., 2006). Interestingly, these two tyrosines also serve as docking sites for Vav1 binding. It is unclear whether one molecule of HS1 can bind simultaneously to Itk and Vav1. This need not be the case, since multiple molecules of HS1 would be present in any given actin-associated signaling complex. Although our *in vitro* data clearly indicate that HS1 and Itk associate via activation-dependent phosphotyrosine-based interactions, these two molecules can be co-immunoprecipitated constitutively from T cell lysates. This constitutive interaction does not appear to involve the SH3 domain of either molecule. This interaction can be explained, in part, by basal tyrosine phosphorylation of HS1, but it probably also involves mutual interactions of these proteins with other T cell signaling molecules. Similar conclusions have been reached for other signalosome proteins, e.g. Itk and SLP-76 (Jordan et al., 2008).

In addition to their similarities with respect to actin dynamics, T cells lacking Itk or HS1 both have defects in Ca^{2+} store release (Gomez et al., 2006; Liu et al., 1998; Schaeffer et al., 1999). In the case of Itk-deficient T cells, the defects in Ca^{2+} store release are well-studied and stem from the requirement for Itk-dependent phosphorylation of PLCγ1. Since HS1 binds to Itk, we considered the possibility that HS1 binding

promotes Itk kinase activity, as has been documented for Slp76 and Itk (Bogin et al., 2007). However, HS1 appears to be dispensable for Itk kinase activity, since HS1-deficient cells show normal kinetics and magnitude of PLCγ1 phosphorylation. Based on these findings, we conclude that HS1 and Itk function to promote Ca^{2+} store release through distinct mechanisms. While Itk phosphorylates PLCγ1 (Bogin et al., 2007), HS1 appears to regulate PLCγ1 function by linking it to the actin cytoskeleton. Interestingly, since Itk functions upstream of HS1 in the actin-regulatory pathway, this suggests that Itk may promote PLCγ1 activation in two ways: via its kinase activity and through SH2 domain-mediated adaptor functions. Further analysis will be required to test this idea and to address the interdependence of these two mechanisms.

Consistent with this dual mode of PLCγ1 activation, Braiman *et al*. (Braiman et al., 2006) have shown that proper PLCγ1 function involves a complex interplay between phosphorylation and association with other signalosome components in signaling microclusters. There is evidence in other cell types that PLCγ1 associates with the actin cytoskeleton, and this association can modulate PLCγ1 activity (Bar-Sagi et al., 1993; Nojiri and Hoek, 2000; Pei et al., 1996; Suzuki and Takahashi, 2001). We now show that T cell activation induces partitioning of PLCγ1 into an F-actin rich fraction, and that the phosphorylated pool of PLCγ1 is enriched in this fraction. $HS1^{-/-}$ T cells show impaired cytoskeletal association of PLCγ1. Moreover, whereas control T cells spreading on anti-CD3-coated surfaces show processive assembly of PLCγ1 microclusters near the periphery, HS1-deficient cells show disorganized microcluster assembly, with newly-formed microclusters overlapping with older ones. The simplest explanation for these

observations is that formation of new, active PLCγ1 microclusters is linked to formation of branched actin filaments at the periphery of spreading T cells. Thus, in HS1-deficient cells, defects in stabilization of lamellipodial protrusions result in perturbation of processive microcluster assembly.

It seems likely that the defects we observe in PLCγ1 dynamics underlie the defects in Ca^{2+} store release, and it will be interesting to explore the molecular mechanisms through which this occurs. Recent studies have led to a paradigm in which microcluster dynamics are mechanistically linked to signal generation and extinction. According to this model, early TCR signaling events mostly take place in newly-formed peripheral microclusters. Depending on the quality of the antigen, signaling can continue in centralized signaling complexes, but centralized complexes are ultimately subject to signal extinction via internalization and degradation (Balagopalan et al., 2007; Barr et al., 2006; Cemerski et al., 2008; Lee et al., 2003). Thus, the defects we observe in PLCγ1 microcluster dynamics might result in aberrant signaling due to premature mixing with proteins responsible for signal extinction. Alternatively, efficient TCR signaling may require the forces associated with myosin-based microcluster centralization (Ilani et al., 2009; Varma, 2008). If so, the spreading defects in HS1-deficient T cells may result in reduced PLCγ1 signaling as a result of diminished tension. Finally, the observed defects in Ca^{2+} store release may arise as a result of altered cell geometry. In a well-spread T cell, much of the endoplasmic reticulum is localized within a short distance of the cell surface, whereas in a poorly-spread T cell, the bulk of the endoplasmic reticulum is situated much further away. Since PLCγ1-induced IP3 production is very short-lived,

typically peaking within a minute of TCR engagement (Olenchock et al., 2006), and activation of IP3 receptors is highly cooperative (Meyer et al., 1988), T cell spreading could serve to promote Ca^{2+} store release by bringing ER-associated IP3 receptors into proximity with sites of IP3 production. Additional investigation will be required to test these possibilities and to explore in more general terms the importance of actin polymerization and cell spreading for T cell signaling.

Chapter 4: Analysis of T cells from HS1-/- mice

Introduction

Mice with germline deletion of HS1 were described by the Watanabe lab in 1995 (Taniuchi et al., 1995). This report showed that $HS1^{-/-}$ mice have normal cellularity in all lymphoid organs. A limited analysis of T cell development and peripheral T cell populations revealed no defects, although negative selection was shown to be impaired. However, these studies were limited in scope. Thymocyte and peripheral T cell population analysis was limited to CD4 and CD8 staining. Activation and memory markers were not examined, nor were regulatory T cells. Functionally, T cells exhibited a mild defect in proliferative responses to anti-CD3, but effector T cell function was not tested. These studies were conducted prior to the appreciation of HS1 as an actin regulatory protein. Moreover, our understanding of T cell biology has advanced considerably since these studies were published. In light of our data demonstrating that HS1 is important for T cell activation (Chapter 2), I have conducted an more extended analysis of the T cell compartment of $HS1^{-/-}$ mice, focusing primarily on responses to TCR engagement.

I have conducted a more in-depth analysis of thymocyte development and peripheral T cell populations, and have confirmed that HS1 is not required for thymocyte development. Peripheral T cell populations also appear normal in $HS1^{-/-}$ mice. I have also tested responses to TCR engagement in several assays. Although CTL function appears normal, loss of HS1 results in reduced IFNγ production and a decrease in the

development of T_H1 cells accompanied by an increase in IL4-producing T_H2 cells *in vitro*. Several reports have described a role for cortactin, the non-hematopoietic homolog of HS1, in migration (Bryce et al., 2005; Huang et al., 1998; Kowalski et al., 2005; van Rossum et al., 2006). Surprisingly, HS1 deficient T cells appear to home normally to lymphoid organs. These data support a role for HS1 in specific TCR signaling pathways.

Results

Analysis of thymocyte development in HS1$^{-/-}$ mice

HS1-deficient mice exhibit normal CD4 and CD8 single- and double-positive thymocyte populations and cell numbers (Taniuchi et al., 1995). To verify and extend these observations, I analyzed thymocytes from wild-type and HS1$^{-/-}$ mice by flow cytometry. There was no difference in the number of thymocytes isolated from either WT or HS1$^{-/-}$ mice. Thymocytes were stained for CD4, CD8, CD44, and CD25 to analyze early development through the double-negative stages. HS1$^{-/-}$ mice had normal percentages of single positive and double positive thymocytes (Figure 4.1a). As shown in Figure 4.1b, the percentages of cells in the DN1 (CD4$^-$ CD8$^-$ CD44$^+$ CD25$^-$), DN2 (CD4$^-$ CD8$^-$ CD44$^+$ CD25$^+$), DN3 (CD4$^-$ CD8$^-$ CD44$^-$ CD25$^+$), and DN4 (CD4$^-$ CD8$^-$ CD44$^-$ CD25$^-$) stages were comparable between WT and HS1$^{-/-}$ samples. To examine NKT cell development, thymocytes were stained for CD3, CD4, CD8, and NK1.1. Comparable percentages of NKT cells were detected in WT and HS1$^{-/-}$ thymi (NK1.1$^+$ CD4$^+$ CD3$^+$) (Figure 4.1c). To examine the efficiency of signaling throughout thymocyte development, surface levels CD5 and CD69, both of which are upregulated in response to

TCR engagement, were examined and found to be comparable between WT and $HS1^{-/-}$ mice (Figure 4.1d and 4.1e). In addition, CD3 levels were comparable between WT and $HS1^{-/-}$ thymocytes (Figure 4.1f). Taken together, these data show that HS1 is dispensable for thymocyte development.

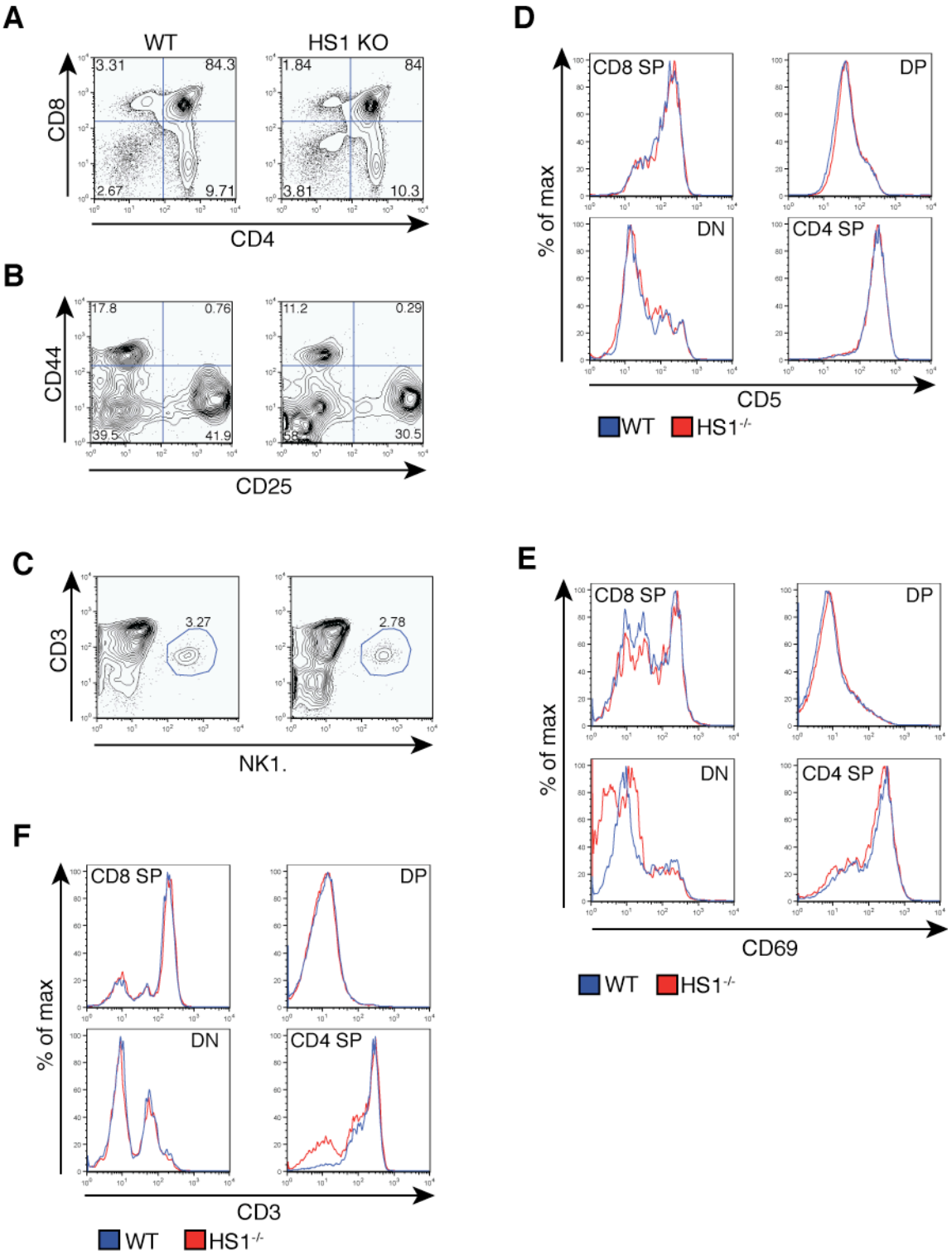

A
WT
HS1 KO
CD8
CD4
3.31
84.3
2.67
9.71
1.84
84
3.81
10.3
B
CD44
CD25
17.8
0.76
39.5
41.9
11.2
0.29
30.5
C
CD3
NK1.
3.27
2.78
D
CD8 SP
DP
DN
CD4 SP
% of max
CD5
WT
HS1-/-
E
CD8 SP
DP
DN
CD4 SP
% of max
CD69
WT
HS1-/-
F
CD8 SP
DP
DN
CD4 SP
% of max
CD3
WT
HS1-/-

Figure 4.1: HS1 is not required for thymocyte development.

Single-cell suspensions from WT or $HS1^{-/-}$ mice were stained for CD4 and CD8 and the indicated cell surface markers. *A*, CD4 and CD8 expression on thymocytes. *B*, Thymocytes stained for CD44 and CD25 were gated on $CD4^-$ $CD8^-$ double-negative cells to analyze early development. *C*, Thymocytes stained for CD3 and NK1.1 were gated on $CD4^+$ cells to analyze NKT cell development. *D*, CD5 expression was analyzed at DN, DP, CD8SP and CD4SP stages. *E*, CD3 expression was analyzed at DN, DP, CD8SP and CD4SP stages. *F*, CD69 expression was analyzed at DN, DP, CD8SP and CD4SP stages.

Normal peripheral T cell populations in HS1 knockout mice

To characterize the peripheral T cell compartment in $HS1^{-/-}$ mice, I began by conducting flow cytometry analysis. Single cell suspensions were prepared from spleens and lymph nodes of WT and $HS1^{-/-}$ mice, and stained for CD4, CD8, CD25, CD69, CD44, and CD62L. $CD4^+$ T cells and $CD8^+$ T cells were present at similar ratios in both WT and $HS1^{-/-}$ mice (Figure 4.2a), as reported previously (Taniuchi et al., 1995). Staining of CD44 and CD62L to distinguish naïve ($CD44^{lo}$ $CD62L^{hi}$) and memory ($CD44^{hi}$ $CD62L^{lo}$) T cells revealed no difference between WT and $HS1^{-/-}$ mice (Figure 4.2b). Similarly, there was no difference in the fraction of freshly isolated T cells expressing the activation markers CD25 or CD69 (Figure 4.2c). To determine if HS1 is required for regulatory T cell development, lymph node and spleen cells were stained with anti-CD4 and anti-CD25, then fixed, permeabilized, and stained with anti-FoxP3. Regulatory T cells ($CD4^+$ $CD25^+$ $FoxP3^+$) were present at equal ratios in WT and $HS1^{-/-}$ mice (Figure 4.3).

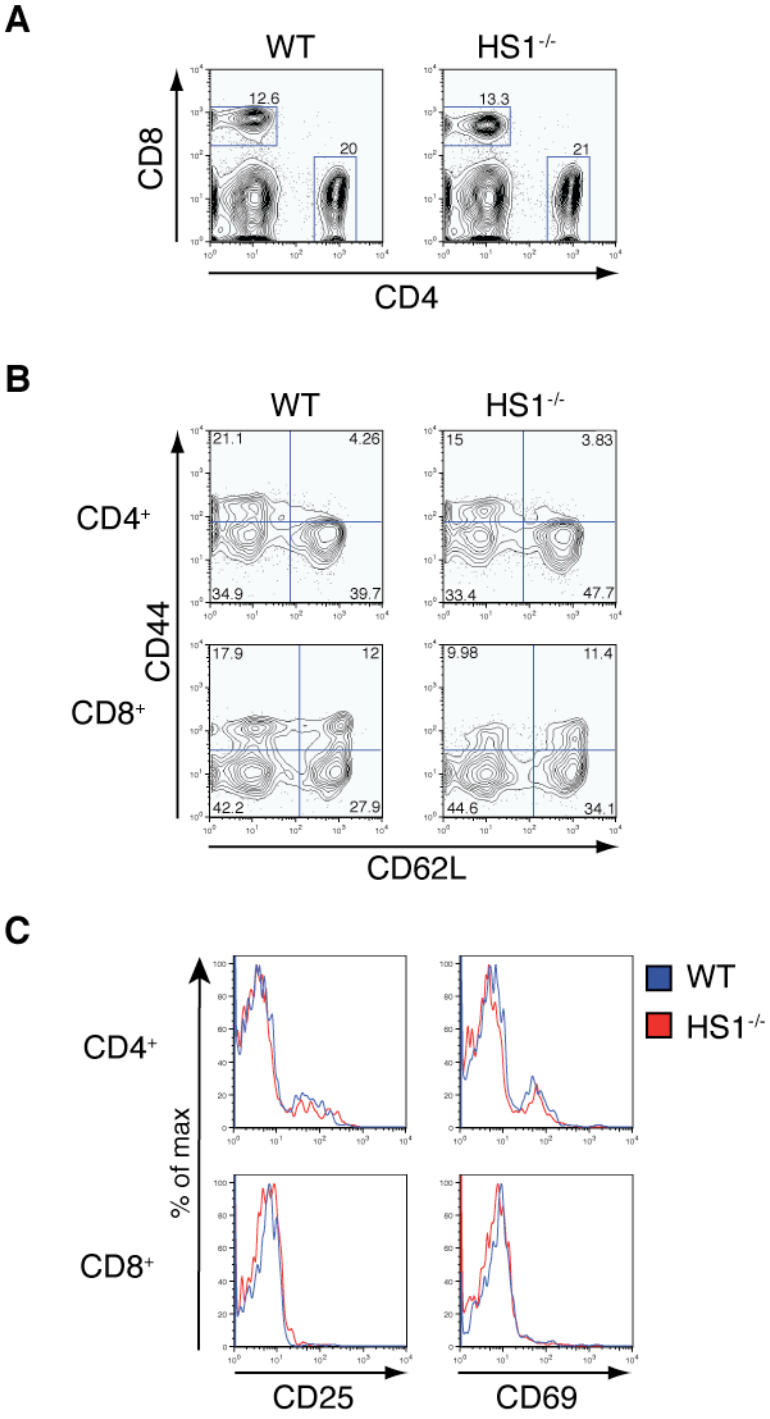

A
WT
HS1-/-
CD8
CD4
12.6
20
13.3
21
B
WT
HS1-/-
CD4+
CD8+
CD44
CD62L
21.1
4.26
34.9
39.7
15
3.83
33.4
47.7
17.9
12
42.2
27.9
9.98
11.4
44.6
34.1
C
WT
HS1-/-
CD4+
CD8+
% of max
CD25
CD69

Figure 4.2: Normal peripheral T cell populations in HS1$^{-/-}$ mice.

Single cell suspensions from spleens and lymph nodes were stained for CD4 and CD8 and the indicated markers. The percentage of cells in each gate is shown. *A*, CD4 and CD8 expression. *B*, Cells gated on CD4^{+} (top) or CD8^{+} (bottom) were analyzed for CD44 and CD62L expression. *C*, Cells gated on CD4^{+} (top) or CD8^{+} (bottom) were analyzed for CD25 (left) and CD69 (right) expression.

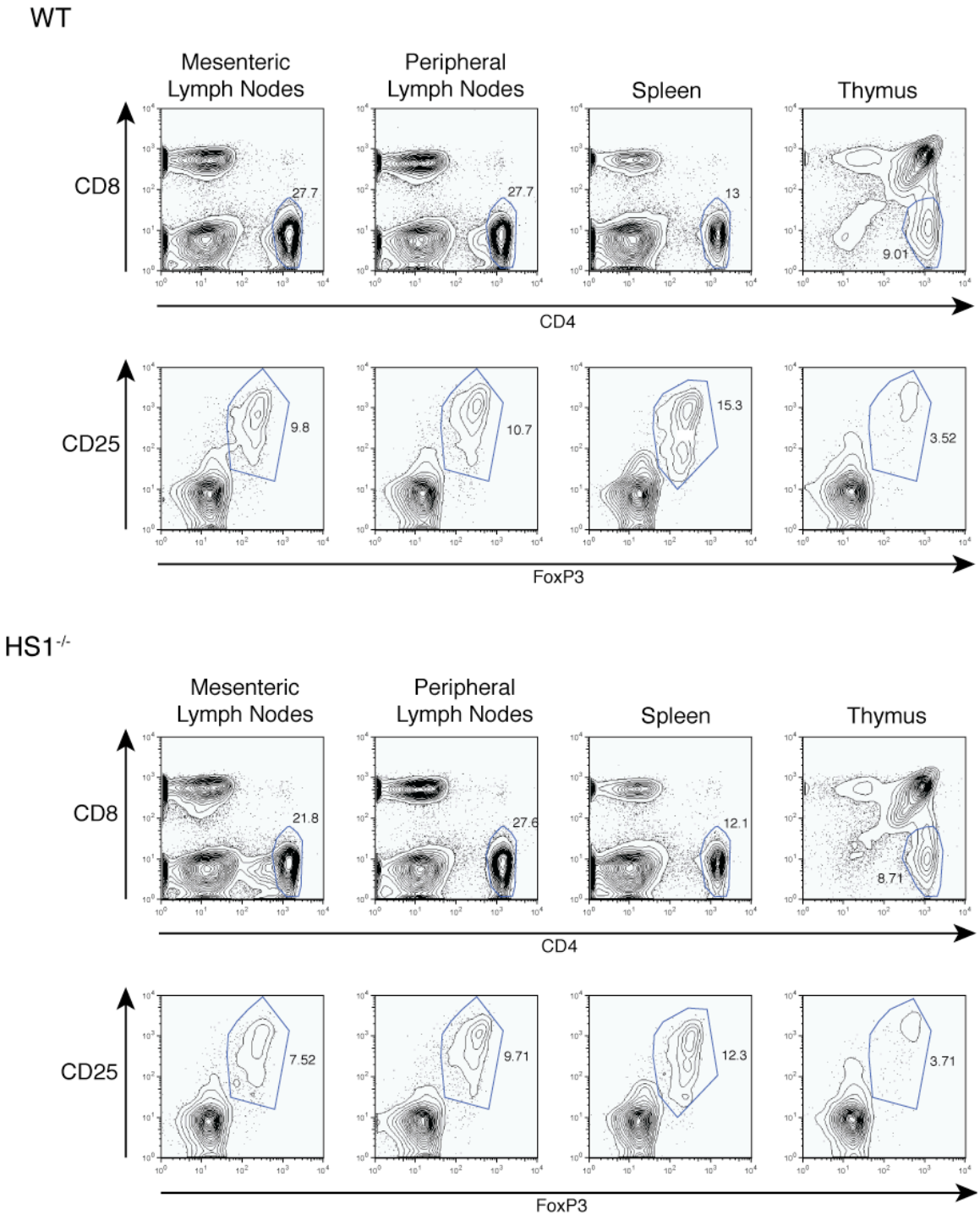

WT
Mesenteric Lymph Nodes
Peripheral Lymph Nodes
Spleen
Thymus
CD8
27.7
27.7
13
9.01
CD4
CD25
9.8
10.7
15.3
3.52
FoxP3
HS1-/-
Mesenteric Lymph Nodes
Peripheral Lymph Nodes
Spleen
Thymus
CD8
21.8
27.6
12.1
8.71
CD4
CD25
7.52
9.71
12.3
3.71
FoxP3

Figure 4.3: HS1 is not required for regulatory T cell development.

Single cell suspensions from mesenteric lymph nodes, peripheral lymph nodes, spleens, and thymi from WT (top) or $HS1^{-/-}$ (bottom) mice were surface-stained for CD4, CD8, and CD25, then fixed, permeabilized, and stained for FoxP3. Treg were identified as $CD25^{+}$ $FoxP3^{+}$ cells within the $CD4^{+}$ gate. The percentage of cells in each gate is shown. Results are representative of two independent experiments.

HS1 in T_H1/T_H2 skewing

$CD4^+$ T cells respond to immunological challenge by differentiating into various effector subsets that secrete different sets of cytokines; each of these subsets is best suited to respond to a different type of infection (Schoenborn and Wilson, 2007). T_H1 cells secrete IFNγ and IL2, leading to the development of a cell-mediated immune response, which is required for the elimination intracellular pathogens such as viruses. T_H2 cells secrete IL4, IL5, and IL13, and promote the development of a humoral immune response, which promotes the clearance of intestinal helminths.

Signaling through both cytokine receptors and through the TCR influences the decision to differentiate to the T_H1 or T_H2 lineage. IL12 and IFNγ can lead to T_H1 differentiation, whereas IL4 promotes T_H2 differentiation. The strength of signal received through the TCR also influences the differentiation process: strong signals that promote robust, long-lived Ca^{2+} responses promote differentiation to the T_H1 lineage, whereas weaker signals, such as those provided by partial agonists, tend to skew T cells to the T_H2 lineage (Constant et al., 1995; Pfeiffer et al., 1995). In light of our data showing defective Ca^{2+} responses in HS1-deficient cells (Chapter 2 and Chapter 3), we hypothesized that HS1-deficient T cells would be less likely to become T_H1 cells and more likely to become T_H2 cells following TCR engagement. However, in Chapter 3, I describe a direct interaction between HS1 and Itk, which suggests an alternate possibility. $Itk^{-/-}$ T cells exhibit significant defects *in vitro* and *in vivo* (Fowell et al., 1999; Miller et

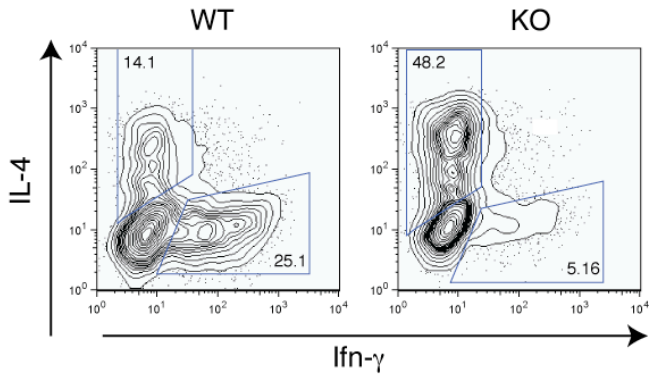
al., 2004; Schaeffer et al., 2001). Together, these results suggest that HS1 deficiency may result in decreased T_H2 skewing downstream of TCR engagement.

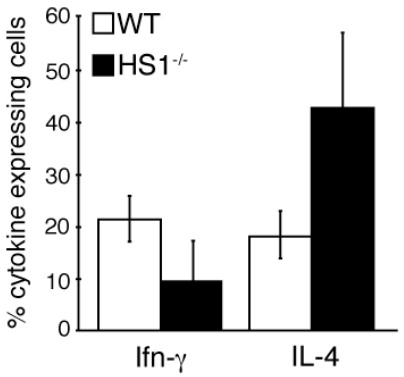
To examine the role of HS1 in helper T cell differentiation and discriminate between the scenarios described above, $CD4^+$ T cells from WT or $HS1^{-/-}$ mice were stimulated *in vitro* on plates coated with anti-CD3 and anti-CD28 for three days, then allowed to proliferate for four days. Cells were then restimulated with PMA and ionomycin for four hours. Brefeldin A was added for the last two hours of culture to prevent cytokine secretion. Cells were then stained with anti-IFNγ and anti-IL4, and analyzed by flow cytometry. As shown in Figure 4.4a**,** HS1-deficient cultures had a higher frequency of IL4-producing cells compared to WT and a lower frequency of IFNγ-producing cells. Although the fraction of cells that skewed to each population varied between experiments, HS1-deficient cells consistently exhibited significantly more T_H2 skewing than did WT cells. These results support the first scenario described above, in which HS1, by promoting a strong Ca^{2+} response, promotes differentiation to IFNγ producing T_H1 cells.

IFNγ signaling plays a key role in the T_H1 differentiation program by inducing production of the T_H1-associated transcription factor Tbet (Lighvani et al., 2001; Mullen et al., 2001; Szabo et al., 2002). Since NF-AT is involved in activation of the *IFNg* promoter (Lee et al., 2004; Schoenborn and Wilson, 2007; Sica et al., 1997; Sweetser et al., 1998), and HS1 deficiency results in reduced NF-AT activation (Chapter 2), I examined IFNγ production by *ex vivo* $CD4^+$ T cells from WT or $HS1^{-/-}$ mice. Cells were stimulated on plates coated with high (1 μg/ml) and low (0.1 μg/ml) doses of anti-CD3

and 1 μg/ml anti-CD28 for three days, after which supernatants were harvested and analyzed by ELISA for IFNγ production (Figure 4.4b). At both high and low doses of anti-CD3, $HS1^{-/-}$ $CD4^{+}$ T cells produced less IFNγ than WT $CD4^{+}$ T cells.

A


WT
KO
14.1
25.1
48.2
5.16
IL-4
Ifn-γ


WT
HS1-/-
% cytokine expressing cells
Ifn-γ
IL-4

B

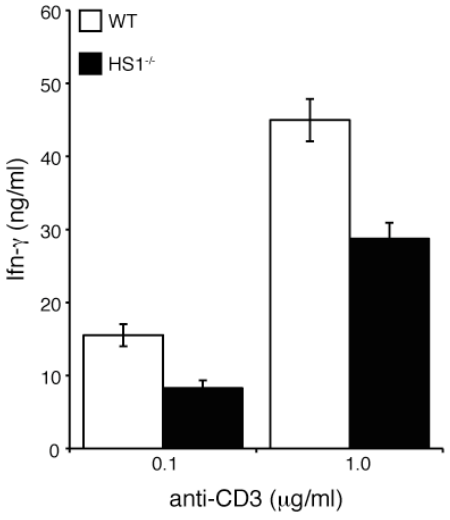

WT
HS1-/-
Ifn-γ (ng/ml)
0.1
1.0
anti-CD3 (μg/ml)

Figure 4.4: HS1-deficient T cells have defects in T_H1 skewing and IFNγ production.

A, CD4+ T cells from WT or HS1-/- mice were stimulated on plates coated with the indicated concentration of anti-CD3 and 1 μg/ml anti-CD28 for three days. Cells were then transferred to fresh plates and allowed to rest for four days. Cells were restimulated with PMA and ionomycin in the presence of brefeldin-A, fixed, and stained for IFN-γ and IL-4, and analyzed by flow cytometry. (Left), Representative flow cytometry plots. The percentage of cells in each gate is shown. (Right), Quantitation of triplicate samples showing the percent of cells that expressed either IFN-γ or IL4. Error bars indicate +/- 1 standard deviation. Results are representative of two independent experiments. *B,* CD4+ T cells from WT or $HS1^{-/-}$ mice were stimulated for three days with plate-bound anti-CD3 and anti-CD28, and supernatants were assayed for IFN-γ by ELISA.

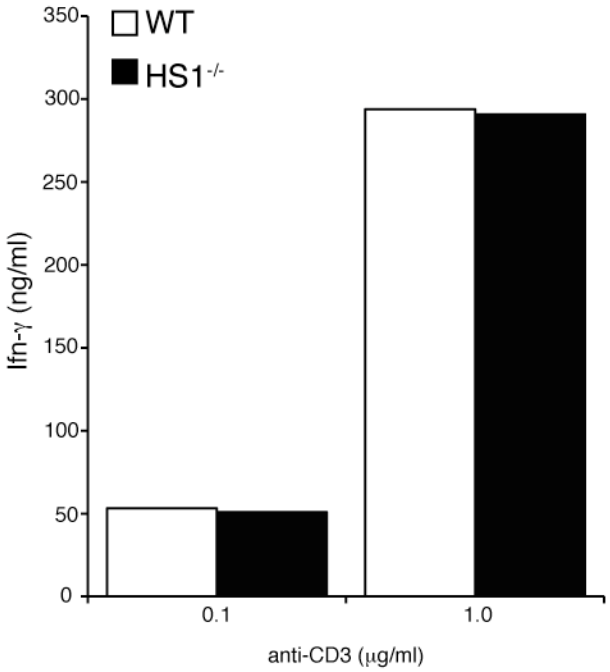
Role of HS1 in cytotoxic T lymphocyte function

Cytotoxic T lymphocytes (CTL) mediate adaptive immunity against intracellular pathogens such as viruses and tumors by lysing infected or malignant cells bearing the appropriate cognate antigen. These cells are also a major source of IFNγ.

To determine whether HS1 is required for CTL function, CTL were generated *in vitro* by stimulating $CD8^+$ T cells isolated by negative selection from lymph nodes from WT or $HS1^{-/-}$ mice (C57Bl/6, H-2^b) on plates coated with anti-CD3 and anti-CD28, in the presence of IL2. After three days of stimulation, these cells were transferred to uncoated plates, and cultured in the presence of exogenous IL2 for 2-3 days. WT and $HS1^{-/-}$ CTL were then assayed *in vitro* for cytotoxicity against allogeneic (H-2^d) ^{51}Cr-labeled P815 target cells. Target cell lysis was equivalent between WT and $HS1^{-/-}$ cells (**Figure 4.5a**), indicating that HS1 is not required for CTL function.

$CD8^+$ T cells produce substantial amounts of IFNγ upon TCR engagement (Szabo et al., 2002). To determine if HS1 is required for IFNγ production by $CD8^+$ T cells, WT or $HS1^{-/-}$ $CD8^+$ T cells were stimulated using plate-bound anti-CD3 and anti-CD28 for three days. As shown in **Figure 4.5b**, WT and $HS1^{-/-}$ $CD8^+$ T cells produced equivalent amounts of IFNγ. Thus, HS1 is not required for efficient IFNγ production by $CD8^+$ T cells.

A


WT
HS1-/-
350
300
250
200
150
100
50
0
Ifn-γ (ng/ml)
0.1
1.0
anti-CD3 (μg/ml)

B

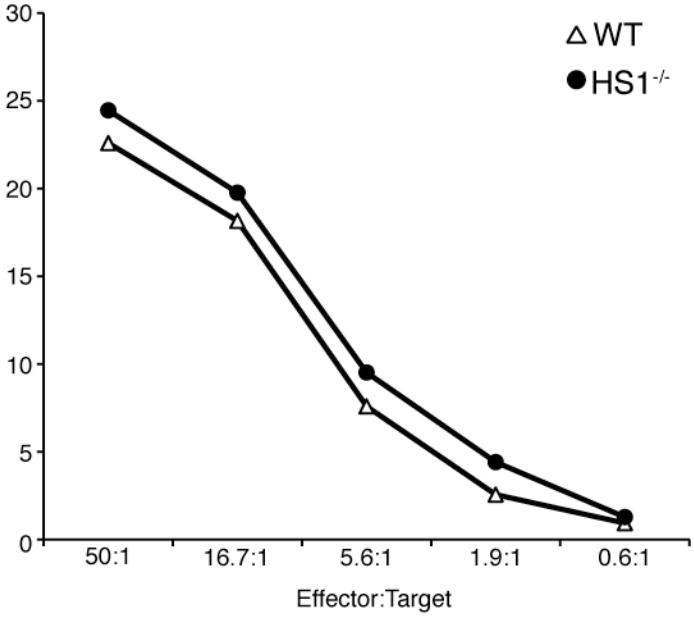

WT
HS1-/-
30
25
20
15
10
5
0
50:1
16.7:1
5.6:1
1.9:1
0.6:1
Effector:Target

Figure 4.5: HS1 is not required for CTL-mediated killing or IFNγ production.

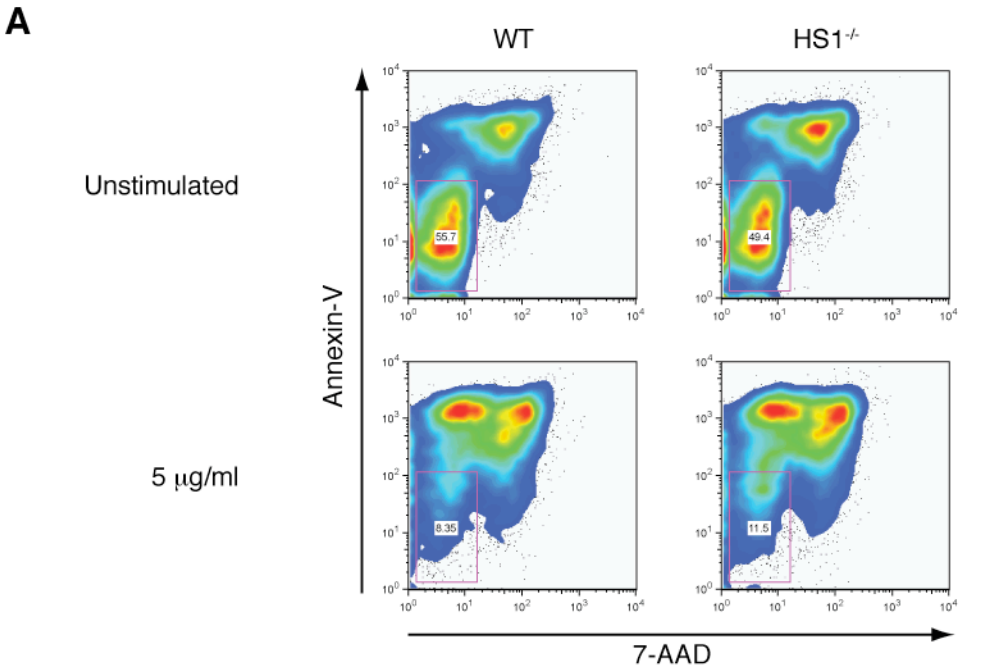
A, CTL generated *in vitro* from wild-type or $HS1^{-/-}$ C57Bl/6 mice were incubated with ^{51}Cr-labeled P815 mouse mastocytoma cells at the indicated effector:target cell ratios. Supernatants were harvested after 5 hours, transferred to a Luma plate, allowed to evaporate overnight, and measured using a TopCount scintillation counter. Results are representative of two independent experiments. *B*, $CD8^+$ T cells from WT or $HS1^{-/-}$ mice were stimulated for three days with plate-bound anti-CD3 and anti-CD28. Supernatants were then assayed by ELISA for IFNγ content.

Activation-induced cell death

Strong signaling through B cell or T cell antigen receptors induced by restimulation shortly after the initial activation of naïve lymphocytes leads to apoptosis through a process termed activation-induced cell death. This process is important for the control of chronically activated T cells, and for downregulation of the immune response (Brenner et al., 2008).

B cells in the peritoneal cavity of HS1-deficient mice are resistant to apoptosis induced by strong crosslinking of the B cell receptor (Taniuchi et al., 1995). Furthermore, two WEHI-231 B cell lines have been isolated that are strongly resistant to AICD. These cells express low levels of HS1, and reexpression of HS1 rescues the death response in one of these lines (Fukuda et al., 1995). Based on these findings, I asked if $HS1^{-/-}$ T cell blasts restimulated *in vitro* were sensitive to AICD.

$CD4^{+}$ T cells from the lymph nodes of WT or $HS1^{-/-}$ mice were stimulated on plate-bound anti-CD3 and anti-CD28 for 3 days, followed by an expansion phase of three days in fresh culture plates in the presence of exogenous IL2. T cells were then harvested, and restimulated on plates coated with varying concentrations of anti-CD3 or left unstimulated. After eight hours, cells were harvested, stained with Annexin-V to detect cells undergoing apoptosis and 7-AAD to detect dead cells, and analyzed by flow cytometry. As shown in **Figure 4.6**, WT and $HS1^{-/-}$ T cells were equally sensitive to AICD at all doses tested. These data demonstrate that HS1 is not required for AICD in mature T cells.


A
WT
HS1-/-
Unstimulated
5 μg/ml
Annexin-V
7-AAD
55.7
49.4
8.35
11.5

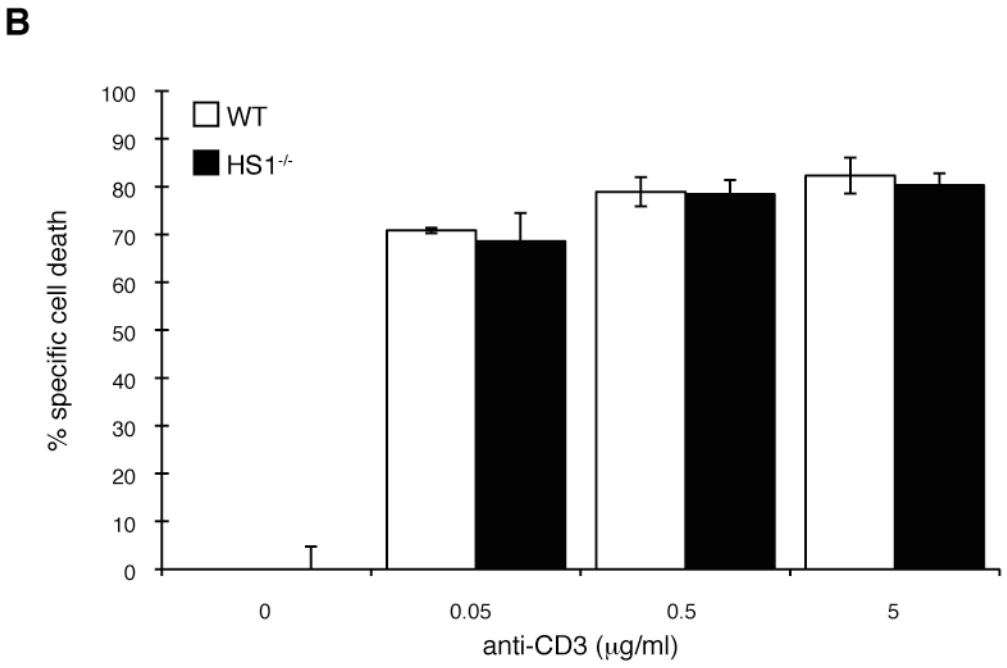

B
WT
HS1-/-
% specific cell death
anti-CD3 (μg/ml)
0
0.05
0.5
5

Figure 4.6: HS1 is not required for AICD in T cells.

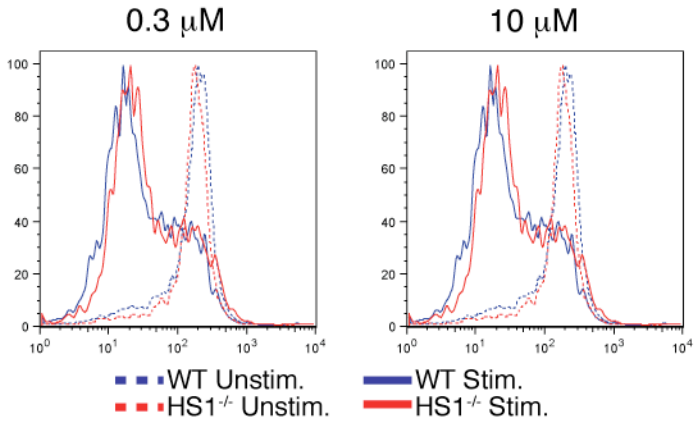
WT or HS1$^{-/-}$ CD4^{+} T cell blasts were stimulated on plates coated with the indicated concentrations of anti-CD3 for eight hours, the harvested, and stained with anti-CD4, 7-AAD, and Annexin-V. *A*, Representative flow cytometry plots. *B*, The percent specific cell death was calculated as $1 - \frac{\%\ Live_{treated}}{\%\ Live_{untreated}} \times 100$. Results are representative of two independent experiments.

TCR Internalization

After stimulation, TCRs are internalized via clathrin-coated pits (Dietrich et al., 1994; Schneider et al., 1999) and degraded via a lysosomal pathway (Valitutti et al., 1997). TCR internalization, an actin dependent process (McGavin et al., 2001) is a critical for the regulation of T cell responses (Ferber et al., 1994; Geisler, 2004; Naramura et al., 2002; Schonrich et al., 1991; Viola and Lanzavecchia, 1996; Zanders et al., 1983). The HS1 homolog cortactin, in conjunction with CD2AP and dynamin family members, is a critical component of the clathrin-dependent endocytic pathway (Cao et al., 2003; Lynch et al., 2003). T cells deficient for CD2AP have severe defects in TCR endocytosis after antigen encounter, and as such are hyperresponsive (Lee et al., 2003).

Based on these observations, I asked whether HS1 is required for TCR endocytosis. AND TCR transgenic $CD4^+$ T cells derived from WT or $HS1^{-/-}$ mice were stimulated *in vitro* with CH27 B cells loaded with varying concentrations of $MCC_{88\text{-}103}$ for 10 minutes to 3 hours. Conjugates were then harvested, stained with anti-Vα11, the α chain of the AND TCR, and analyzed by flow cytometry. As shown in **Figure 4.7**, T cells lacking HS1 were able to downregulate surface TCR at rates equivalent to WT T cells, and responded equally well to high and low doses of peptide. These data suggest that HS1 does not play a role in downregulation of surface TCR upon stimulation.

A


0.3 μM
10 μM
WT Unstim.
HS1-/- Unstim.
WT Stim.
HS1-/- Stim.

B

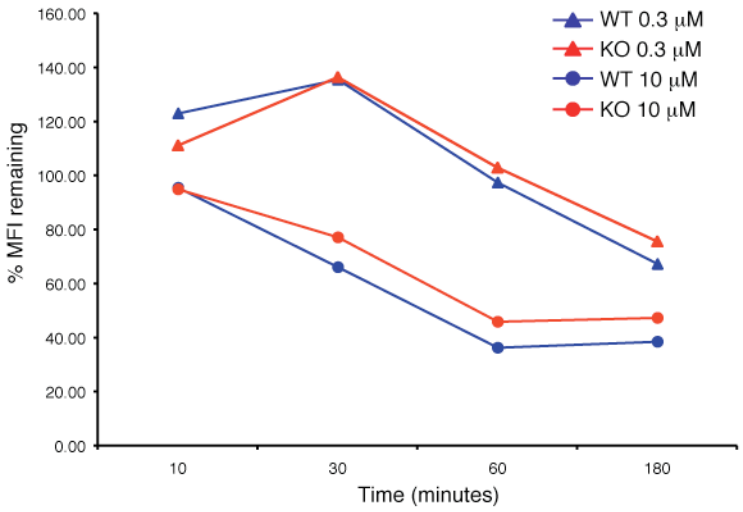

WT 0.3 μM
KO 0.3 μM
WT 10 μM
KO 10 μM
% MFI remaining
Time (minutes)

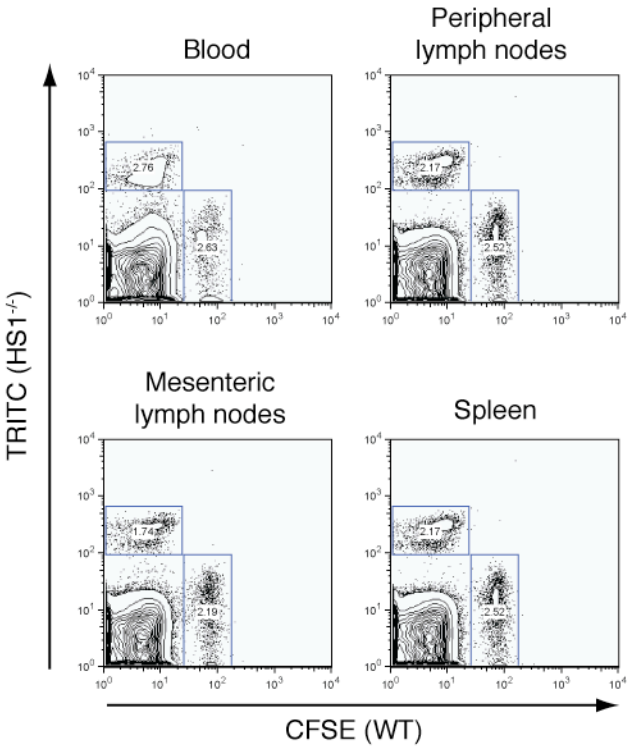
Figure 4.7: HS1 is not required for TCR internalization.

T cells from WT or HS1$^{-/-}$ AND TCR transgenic mice were stimulated with CD27 B cells loaded with 0.3 or 10 μM $MCC_{88\text{-}103}$ peptide for ten minutes to three hours, as indicated. Cells were stained for CD4 and Vα11, and analyzed by flow cytometry. (A), Representative flow cytometry plots. (B), TCR internalization was calculated as the percent MFI remaining, compared to unstimulated cells.

Normal homing of HS1$^{-/-}$ T cells to lymphoid organs

Several studies have identified a role for cortactin, the non-hematopoietic homolog of HS1, in cell migration (Lai et al., 2009; Patel et al., 1998). Actin regulatory proteins such as WASp and WIP, which constitutively interact, play important roles in lymphocyte migration (Gallego et al., 2006; Haddad et al., 2001). Therefore, we asked if HS1 is required for T cell homing to lymphoid organs. Single-cell suspensions from lymph nodes and spleens from WT or HS1$^{-/-}$ mice were labeled with either CFSE or TRITC, and injected at a 1:1 ratio into WT C57BL/6 recipients. Injected cells were allowed to migrate overnight, after which recipients were euthanized. Liver, lungs, blood, spleen, and peripheral and mesenteric lymph nodes were analyzed by flow cytometry for the presence of labeled injected T cells. As shown in Figure 4.8, WT and HS1$^{-/-}$ cells were able to home equally well to all tissues examined, suggesting that HS1 is dispensable for T cell homing to lymphoid organs in the absence of an inflammatory response.

A


Blood
Peripheral lymph nodes
Mesenteric lymph nodes
Spleen
TRITC (HS1-/-)
CFSE (WT)
2.76
2.63
2.17
2.52
1.74
2.19
2.17
2.52

B

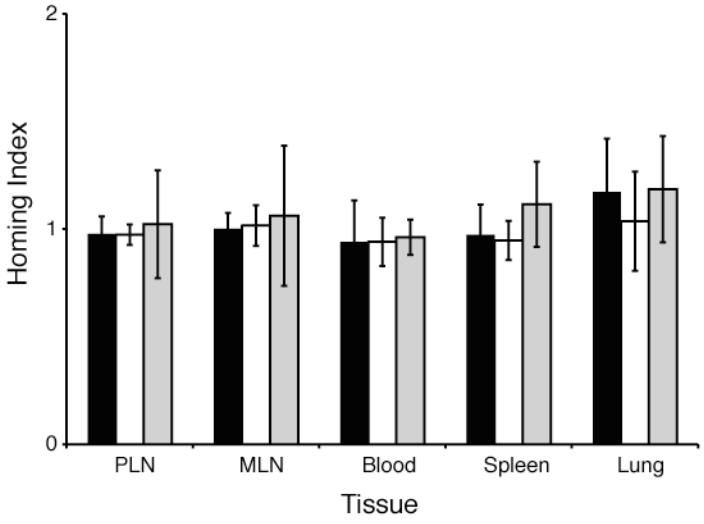

Homing Index
2
1
0
PLN
MLN
Blood
Spleen
Lung
Tissue

Figure 4.8: HS1 is not required for naïve T cell migration to lymphoid organs.

Single cell suspensions from spleens and lymph nodes of WT or HS1$^{-/-}$ mice were stained with CFSE (WT) or TRITC (HS1$^{-/-}$) and mixed at a 1:1 ratio. 2x10^7 cells were injected into each WT recipient. A small sample of input cells was saved as a control for variation in WT:HS1$^{-/-}$ ratios. After twenty-four hours, recipients were sacrificed, and tissues were harvested, stained for CD4 and CD8, and analyzed by flow cytometry. (A), Representative flow cytometry plots. (B), Homing index was calculated according to the following equation: $\frac{(\%WT/\%KO)_{in\ tissue}}{(\%WT/\%KO)_{input}}$. Data are representative of two independent experiments.

Discussion

Although HS1 seems to be important for efficient TCR signaling in mature T cells, the TCR signaling pathways that promote thymocyte maturation seem to be independent of HS1. Alternatively, thymocyte signaling might be impaired only to the extent that negative selection is reduced, as described in (Taniuchi et al., 1995). It may be that positive selection is also impaired in the absence of HS1, such that the TCR repertoire is skewed towards a more auto-reactive set. However, we have not observed, and have not seen any reports detailing, a tendency towards autoimmune disease in $HS1^{-/-}$ mice. If selection is altered in $HS1^{-/-}$ thymocytes, it may be that additional contributing factors are necessary to initiate an autoimmune response. To that end, it might be interesting to breed $HS1^{-/-}$ mice to a model of autoimmunity (*e.g., SLE1/2/3)* to examine changes in the kinetics of development or magnitude of autoimmune disease.

Peripheral T cell populations also appear normal in $HS1^{-/-}$ mice, in agreement with the finding that HS1 is dispensable for T cell development. Although regulatory T cell development appears normal, these cells may exhibit reduced function. WASp is not strictly required Treg development, several studies have now established that Treg from $WASp^{-/-}$ mice and from WAS patients are functionally impaired, leading to an increased risk of autoimmune disease (Adriani et al., 2007; Humblet-Baron et al., 2007; Maillard et al., 2007; Marangoni et al., 2007). This may be independent of the actin-regulatory function of these proteins, because WT Treg do not efficiently polymerize actin at the IS.

Negative selection in HS1$^{-/-}$ mice was originally studied in the HY TCR transgenic system, in which T cells specific for the male antigen are deleted in male mice. Expression of TCRα occurs abnormally early in this model system, leading to aberrant thymocyte development and selection. Expression of the HY TCR α chain during the DP stage promotes a development and selection pattern that more closely reflects that seen in WT C57BL/6 mice (Baldwin et al., 2005). Thus, the negative selection phenotype in HS1$^{-/-}$ mice may have been overstated. To address this issue, negative selection could be studied in a superantigen model system, in which the presence of endogenous superantigens leads to the deletion of thymocytes expressing specific Vβ chains (Jordan et al., 2008). Thymocyte CD5 levels correlate directly with TCR signal strength (Azzam et al., 1998). That I did not observe alterations in CD5 levels at any stage of development suggests that HS1 does not affect thymocyte signal strength.

Of all the phenotypes examined, the most striking is a significant decrease in the development of IFNγ-producing cells after *in vitro* stimulation. The reason for a defect in T_H1 differentiation *in vitro* in HS1$^{-/-}$ T cells is not clear. Several possibilities exist. HS1-deficient T cells exhibit a defect in Ca^{2+} signaling (Chapters 2 and 3). As discussed above, reduced Ca^{2+} signaling is associated with T_H2 responses. Stimulation of naïve T cells leads to the Ca^{2+}-dependent activation of the NF-AT transcription factor, which is partly responsible, in concert with NFκB and AP1, for the activation of the *IL2* and *IFNg* promoters (Schoenborn and Wilson, 2007). The Ca^{2+} signaling defect leading to reduced NF-AT activation in HS1$^{-/-}$ T cells (Chapter 2) may lead to reduced production of IFNγ; this, in turn, may reduce the efficiency with which HS1$^{-/-}$ T cells differentiate to T_H1

cells. Differentiated T_H1 cells, due to their higher usage of the Ca^{2+} signaling pathway (Sloan-Lancaster et al., 1997), may also be more dependent on HS1 for T_H1 recall responses. That is to say, HS1-deficient T cells may be able to differentiate to the T_H1 lineage normally, but may fail to produce IFNγ normally upon restimulation. However, this scenario is less likely because to measure detect intracellular cytokines, cells were restimulated with PMA and ionomycin, which stimulate components downstream in the signaling cascade compared to where HS1 is thought to act.

HS1 is required for efficient IFNγ production by freshly isolated $CD4^+$ T cells (**Figure 4.4**). Recent mathematical modeling studies have suggested that IFNγ is key to the initiation of T_H1 differentiation by inducing expression of the T_H1-associated transcription factor Tbet (Schulz et al., 2009). Thus, without additional signals from other cells (*e.g.*, cytokine signals derived from APCs or other bystander cells), inefficient IFNγ production by $HS1^{-/-}$ T cells in response to TCR and CD28 ligation may lead to inefficient differentiation to the T_H1 lineage. HS1 is required for full activation of the NF-AT and NF-κB transcription factors downstream of TCR engagement (Chapter 2 and (Gomez et al., 2006)), both of which are involved in transcription of the *IFNg* gene (Schoenborn and Wilson, 2007; Sica et al., 1997). It would therefore be important directly characterize the activation of the *IFNg* promoter with respect to these as well as other key transcription factors in $HS1^{-/-}$ cells.

Other actin regulatory proteins have been shown to influence helper T cell skewing and cytokine production. $CD4^+$ T cells from Wiskott-Aldrich syndrome patients exhibit severe defects in T_H1 cytokine production in response to TCR stimulation, even

after culture in a T_H1 skewing environment (Trifari et al., 2006). These cells, as well as $CD4^+$ T cells from $WASp^{-/-}$ mice (Cannon and Burkhardt, 2004) exhibit defects in NF-AT nuclear translocation and maintenance. This stands in contrast to T cells lacking Vav1, which preferentially produce T_H1-associated cytokines at the expense of T_H2 cytokines (Tanaka et al., 2005). Disruption of the WAVE2 complex through loss of Hem1 leads to normal IFNγ production, but substantially increased production of IL17 These varied effects underscore the complexity of the signaling pathways leading to cytokine production and differentiation.

Ultimately, it will be important to test how well $HS1^{-/-}$ mice respond to immune challenge using a variety of models. The original report describing the $HS1^{-/-}$ mice demonstrated normal antibody responses to the T-dependent antigen NP-chicken gammaglobulin, but slightly reduced responses to the T-independent antigen TNP-Ficoll (Taniuchi et al., 1995). These experiments suggest that B cell function *in vivo* is not grossly impaired. $HS1^{-/-}$ $CD4^+$ T cells produce less IFNγ upon initial stimulation, and are less likely to become IFNγ-producing T_H1 effector cells, compared to WT $CD4^+$ T cells. Therefore, it will be interesting to test how well these mice respond to pathogens, such as *L. major*, that require a robust T_H1 response for clearance. The reduction in T_H1 cells accompanies an increase in the frequency of IL4-producing T_H2 cells. This may result in improved clearance of T_H2 pathogens, but may also increase susceptibility to T_H2-mediated disease, such as allergy, asthma, and colitis. Therefore, testing reactions to these conditions would also help determine if HS1 is a factor in these diseases. A role for HS1 has not been described in other leukocytes. However, studies in progress in our lab

suggest that antigen presentation by dendritic cells requires HS1. Thus, any *in vivo* studies would require careful design and interpretation. These may involve use of adoptive transfer experiments to generate and study mice that lack HS1 in specific cell types.

The data presented here suggest that HS1 is dispensable for CTL killing. In contrast, the Cooper lab has reported that human NK cell lines lacking HS1 fail to kill target cells, likely due to defects in NK-target cell conjugate formation (Butler et al., 2008). Although it is possible that the discrepancy between my data and those of the Cooper lab are attributable to the species difference, it is more likely that they are due to differences between the way T cells and NK cells employ the actin regulatory machinery. Indeed, the Wülfing group has shown that mild disruption of actin dynamics using low doses of jasplakinolide impairs the ability of NK cells, but not CTL, to kill target cells (Wulfing et al., 2003). The reason for this apparent difference in sensitivity to actin disruption between CTL and NK cells is unclear. The signals downstream of the TCR may be more robust (*i.e.*, less sensitive to mild perturbations) compared to those downstream of NK cell activating receptors. These studies were conducted using high-dose peptide for P14 TCR transgenic CTL or high dose anti-CD3 for polyclonal CTL. Perhaps studies using lower dose or weaker agonists might uncover a situation in which CTL are sensitive to mild disruptions of actin dynamics or loss of HS1.

My data suggest that HS1 is not required for T cell AICD. This stands in contrast to the finding that HS1-deficient WEHI-231 cells (Fukuda et al., 1995) and peritoneal B cells (Taniuchi et al., 1995) are resistant to AICD. The reason for the apparent difference

between B and T cell requirements for HS1 in AICD is not known; however, in addition to differences in the signaling pathways between the two cell types, it is possible that the different experimental approaches contribute. The B cell experiments described by (Taniuchi et al., 1995) were conducted *in vivo*, whereas my studies were conducted by *in vitro* stimulation of $CD4^+$ T cell blasts. Alternatively, HS1 may play a role in AICD only at certain developmental stages. WEHI-231 cells are an immature B cell line, representing a stage at which B cells undergo negative selection—strong signaling through the B cell receptor induces apoptosis to eliminate autoreactive B cells. Similar to this, $HS1^{-/-}$ mice exhibit a defect in negative selection of thymocytes in the HY TCR transgenic system (Taniuchi et al., 1995). Together with data presented here, this suggests that the role of HS1 in AICD is limited to its role in facilitating antigen receptor signaling in developing lymphocytes.

Several studies have demonstrated that cortactin interacts with components of the endocytic machinery, including Dynamin 2 and CD2AP, and have shown a requirement for these interactions in endocytosis (Cao et al., 2003; Lynch et al., 2003; McNiven et al., 2000; Sauvonnet et al., 2005). Moreover, preliminary data from our laboratory have demonstrated a role for HS1 in receptor-mediated endocytosis in dendritic cells (not shown). It was therefore surprising to find that T cells lacking HS1 downregulate surface TCR normally. These experiments were conducted on T cells homozygous for the AND transgene. Therefore, it is possible that developmental abnormalities resulting from premature TCR expression in developing thymocytes compensate for the lack of HS1 in these cells. These studies should be repeated on T

cells hemizygous for the AND transgene, or on polyclonal T cells to rule out the possibility that these data are artifactual.

Jurkat cells depleted of Dynamin 2 by RNAi internalize surface TCR normally after antibody stimulation, in spite of a severe defect in antigen receptor-induced actin polymerization at the IS. The authors suggest that a more physiological stimulus (*i.e.*, peptide/MHC) might reveal a role for Dynamin 2 in TCR internalization (Gomez et al., 2005). Together with my results, these data suggest that the endocytic machinery responsible for downregulating surface TCR upon antigen encounter differs from the cortactin/dynamin system found in other cell types.

Data presented here demonstrate that HS1 is not required for homing to lymphoid organs (**Figure 4.7**). This is somewhat surprising, considering abundant data in non-hematopoietic cell types demonstrating that cortactin is important for regulating cell migration (Huang et al., 2008; Lai et al., 2009; Patel et al., 1998; van Rossum et al., 2006). Moreover, in NK cells, HS1 is required for efficient chemotaxis to SDF-1α, a CXCR4 ligand, and for LFA1-mediated adhesion (Butler et al., 2008). More work is necessary to determine whether HS1 is important for chemokine receptor or integrin function in T cells.

Chapter 5: Discussion

Summary

Regulation of cytoskeletal dynamics is a key component of T cell activation. Early experiments showed that disruption of actin filaments using pharmacological agents can severely impair several aspects of T cell function. This includes interaction with APCs, early signaling events, cytokine production, and killing of target cells by CTL. Mutation in actin regulatory proteins can result in disease, as highlighted by the severe the immunodeficiency Wiskott-Aldrich syndrome. More recently, investigators have begun to tease apart the role and regulation of the actin cytoskeleton in lymphocytes by examining the contributions of individual actin regulatory proteins.

We have examined the role and regulation of HS1 in T cell activation, under the hypothesis that efficient signal transduction downstream of the TCR would depend on HS1. HS1 is a hematopoietic lineage-restricted protein that undergoes tyrosine phosphorylation upon immunoreceptor ligation. It has been shown to regulate the actin cytoskeleton through Arp2/3 complex. Our data show that HS1 is important for the stabilization of F-actin-rich structures, and that it acts as an adaptor protein that can interact with multiple signaling molecules upon T cell activation. HS1 interacts directly with Vav1 and stabilizes it at the IS. HS1 recruitment to the IS depends on Itk, with which it also interacts directly. We show that HS1-deficient T cells have defects in IL2 production stemming from inefficient Ca^{2+} responses. This is likely to be due to regulation of PLCγ1 cytoskeletal association and microcluster dynamics by HS1.

We have also examined the role of HS1 on other aspects of T cell function using $HS1^{-/-}$ mice. Thymocyte development proceeds normally, and peripheral T cell populations are grossly normal based on their expression of characteristic cell-surface markers. $HS1^{-/-}$ $CD8^{+}$ T cell function appears to be normal, but $HS1^{-/-}$ $CD4^{+}$ T cells exhibit defects in IFNγ production upon initial TCR stimulation, and are subsequently less likely to become IFNγ-producing T_H1 effector cells. Here, I discuss the overall significance of these data and potential new avenues for research.

Why does T cell development proceed normally in the absence of HS1?

The loss of HS1 in mice does not result in any detectable perturbations in T cell development or in peripheral populations (Chapter 4). Moreover, $HS1^{-/-}$ mice do not develop any detectable autoimmune disease that would suggest significant defects in negative selection. Developing thymocytes pass through several developmental checkpoints that require TCR signaling, yet HS1 is seemingly dispensable for these processes. Another actin regulatory protein that is essential for T cell activation, WASp, also appears to be dispensable for thymocyte development (Snapper et al., 1998). It is not known why these proteins, which are essential for efficient signaling in peripheral T cells, are seemingly dispensable for thymocyte development. It may be that the signaling pathways in mature T cells in which these proteins participate are different in developing thymocytes.

In addition to differences in signaling pathways between mature and developing T cells, compensatory mechanisms may be at play: one actin regulatory protein may offset

the loss of another. It is possible that thymocytes express low levels of cortactin, which might compensate for loss of HS1. However, we have not detected cortactin mRNA in WT or $HS1^{-/-}$ T cells or in mouse thymus (Chapter 2 and (Gomez et al., 2006)). Compensation between homologous proteins has been demonstrated for WASp. Although mice lacking either WASp or N-WASp exhibit normal thymocyte development, loss of both proteins results in severe defects in thymocyte development and function (Cotta-de-Almeida et al., 2007). Compensation may also exist between non-homologous proteins. It is possible that the activity of WASp masks a role for HS1 in thymocyte development, and vice-versa. To resolve these issues, it would be necessary to generate $HS1^{-/-}$ x $WASp^{-/-}$ mice. It seems likely that such a model would reveal cooperativity or compensation between the two proteins.

In chapter 3, I discuss a direct biochemical and functional interaction between HS1 and Itk in the pathway leading to actin polymerization. This leads one to ask if HS1-deficient T cells or mice resemble Itk-deficient T cells or mice in other respects. Itk deficiency results in the abnormal development of memory-like $CD8^+$ T cells in the periphery and deficient $CD8^+$ T cell responses (Atherly et al., 2006a; Atherly et al., 2006b; Broussard et al., 2006; Hu et al., 2007). HS1 deficiency does not result in the development of these abnormal cells (Chapter 4). Moreover, Itk is required for efficient T_H2 responses (Fowell et al., 1999; Miller et al., 2004), whereas HS1 is required for efficient T_H1-associated cytokine production (Chapter 4). These results highlight the complexity of T cell signaling pathways. Although we can detect a constitutive interaction between HS1 and Itk in T cell blasts, the phenotypic differences between

$HS1^{-/-}$ and $Itk^{-/-}$ mice and T cells make it clear that they participate in different aspects of T cell function.

How does HS1 influence helper T cell differentiation?

We have found that $HS1^{-/-}$ T cells produce reduced amounts of IFNγ, and subsequently are less likely to become T_H1 effector cells, compared to WT T cells (Chapter 4). Signals that induce potent Ca^{2+} responses favor differentiation to T_H1 cells, whereas signals associated with weak Ca^{2+} responses, such as altered peptide ligands or low peptide dose, tend to favor the development of T_H2 cells (Constant et al., 1995; Pfeiffer et al., 1995). Moreover, prolonged NF-AT activity has been associated with increased IFN-γ production and a concomitant decrease in IL4 production (Porter and Clipstone, 2002). Thus, by promoting increased Ca^{2+} flux responses (Chapter 2 and Chapter 3) and therefore sustained NF-AT activity, HS1 may influence IFNγ production, and thus helper T cell differentiation. Thus, in this context, HS1 may function, at least in part, by modulating signal strength. Although this work has not addressed whether the effect of HS1 on IFNγ production and helper T cell differentiation involves its role in regulating actin dynamics, this seems likely to be the case. Disruption of actin dynamics using cytochalasin D results in decreased IFNγ production. The effect is most pronounced when the drug is added 10-60 minutes after APC encounter and is substantially blunted when added later (Valitutti et al., 1995).

Our work with Vav1 (Chapter 2) and PLCγ1 (Chapter 3) shows that HS1 is important for the organization of signaling molecules at the IS downstream of TCR

engagement. In Chapter 4, I show that HS1 is required for the efficient development of IFNγ-producing T_H1 cells. Work from the Glimcher group has demonstrated a correlation between cytokine receptor localization to the IS and helper T cell differentiation (Maldonado et al., 2004). The IFNγ receptor, but not the IL4 receptor, localizes to the IS upon TCR engagement. The IFNγ receptor is more likely to redistribute to the IS in T cells isolated from T_H1-prone C57Bl/6 mice than in T cells from T_H2-prone BALB/c mice. Moreover, this process is can be inhibited by the addition of IL4. More recent work from this group has extended these findings to signaling events downstream of the IFNγ receptor: STAT1 localization mirrors that of IFNγ receptor and IL4 signaling impairs this process (Maldonado et al., 2009). The signaling and cell biological mechanisms that control IFNγ receptor redistribution have not been elucidated. However, it is conceivable that actin-dependent processes regulate the localization of cytokine receptors and downstream signaling molecules. Although a decrease in IFNγ production associated with loss of HS1 is likely to contribute to the observed reduction in the development of IFNγ producing effector cells, abnormal IFNγ receptor redistribution may also play a role.

IFNγ is preferentially secreted into the immunological synapse, whereas IL4 secretion appears to be multidirectional (Huse et al., 2006). The mechanisms controlling cytokine secretion are not well understood. However, actin-dependent processes may contribute to these events: WASp is required for IFNγ secretion, but not for its production (Morales-Tirado et al., 2004). This suggests that a defect in secretion of IFNγ by freshly isolated $HS1^{-/-}$ T cells may underlie the phenotype observed by ELISA (Chapter 4). It

should be noted that this hypothesis is not mutually exclusive with the idea that HS1 regulates IFNγ at the level of promoter activation.

Is there a role for HS1 in endocytosis?

We have found that HS1 is dispensable for TCR internalization following APC encounter (Chapter 4). It has been shown that TCRs are internalized via clathrin-coated vesicles (Boyer et al., 1991; Telerman et al., 1987). This involves the phosphorylation of clathrin heavy chain (Crotzer et al., 2004), a process implicated in clathrin-mediated endocytosis (Stoddart et al., 2002; Wilde et al., 1999). T cells lacking CD2ap, a component of the endocytic machinery, exhibit substantial defects in TCR internalization (Lee et al., 2003). In contrast, Jurkat cells suppressed for Dynamin 2, another component of the endocytic machinery, downregulate surface TCR normally (Gomez et al., 2005). Cortactin has been shown to interact with these components of the endocytic machinery and to be required for efficient endocytosis (Cao et al., 2003; Lynch et al., 2003; McNiven et al., 2000; Sauvonnet et al., 2005). Moreover, preliminary data from our lab has demonstrated a requirement for HS1 in receptor-mediated endocytosis in dendritic cells (not shown). It is therefore surprising that HS1 is not required for TCR internalization. It may be that a protein other than HS1 fulfills the role of cortactin in endocytosis in T cells.

Even if it is dispensable for TCR endocytosis, HS1 may play a role in the internalization of other surface receptors, *e.g.*, the IL2 receptor. In a system involving ectopic expression of the β chain of the IL2 receptor (IL2Rβ, CD122) in an epithelial cell

line, it was found that cortactin, together with dynamin, is required for IL2Rβ internalization in a clathrin-independent manner (Grassart et al., 2008). The same group also found that endocytosis of the common gamma chain (γc), a component of the IL2, IL4, IL7, IL9, IL15, and IL21 receptors, requires cortactin (Sauvonnet et al., 2005). Because these studies were conducted in a cell line that does not normally express these receptors, it would be necessary to validate these results in a more physiologically relevant system, and to determine if HS1 plays a similar role.

Why is CTL function independent of HS1?

Given the role of HS1 in efficient IFNγ production in $CD4^+$ T cells, it is striking that HS1 is not required for production of IFNγ in $CD8^+$ T cells. Similarly, considering work showing a requirement for actin in cytotoxicity (Cerottini and Brunner, 1972; O'Rourke et al., 1991; Plaut et al., 1973; Wulfing et al., 2003), it is surprising that $HS1^{-/-}$ CTL kill target cells efficiently (Chapter 4). In $CD4^+$ T cells, IFNγ production and differentiation to the T_H1 lineage, as well as killing by NK cells, depend on the transcription factor T-bet (Mullen et al., 2001; Szabo et al., 2002). In contrast, IFNγ production and killing by $CD8^+$ T cells instead relies on the related transcription factor Eomesodermin (Pearce et al., 2003). Given that I find a defect in IFNγ production only in $CD4^+$ T cells and no defect in killing by $HS1^{-/-}$ $CD8^+$ T cells, it is possible that HS1 may be required for the induction of T-bet but not Eomesodermin. No role for HS1 in cytokine production by NK cells has been described.

Pharmacological studies have demonstrated that the actin cytoskeleton is important for cytotoxicity (Cerottini and Brunner, 1972; O'Rourke et al., 1991; Plaut et al., 1973; Wulfing et al., 2003). However, in these studies, inhibition of CTL-mediated killing required relatively high drug doses. Interestingly, Wülfing *et al*. showed that killing by NK cells, but not by $CD8^+$ T cells, is sensitive to low doses of jasplakinolide, a drug that stabilizes branched actin filaments (Wulfing et al., 2003). Our data show that HS1 is not absolutely required for actin polymerization in T cells, and instead primarily functions to stabilize existing F-actin (Chapter 2). HS1 deficiency results in defects in cytotoxicity in NK cells (Butler et al., 2008). Moreover, while NK cells from WAS patients exhibit clear defects in cytotoxicity (Orange et al., 2002), no such defect has been reported for CTL. No clear explanation has arisen for this difference between T cells and NK cells. While our data suggest that HS1 deficiency results in unstable immunological synapses, it has been shown that CTL can kill efficiently even in the absence of a stable IS (Purbhoo et al., 2004). While IS organization may be important for signaling events, taken together, all these studies point to fundamental differences in the cell biology of CTL and NK cell killing. This is an area that warrants future study.

Is there a role in T cells for HS1 in integrin or chemokine receptor function?

In addition to describing a role in NK cell-mediated cytotoxicity, the Cooper lab study has implicated HS1 in NK cell adhesion and migration processes (Butler et al., 2008). This study showed that HS1-deficient NK cells exhibit defects in adhesion to

target cells and in migration to CXCL12 (SDF1α), a CXCR4 ligand. Moreover, several studies have implicated cortactin in migration of non-hematopoietic cells (Bryce et al., 2005; Huang et al., 1998; Kowalski et al., 2005; van Rossum et al., 2006). Our laboratory has also shown that the tyrosine kinase c-Abl, which can interact with HS1 in T cells, is required for chemokine-induced migration (Huang et al., 2008). In contrast to these results, data presented in Chapter 4 indicate that HS1 is not required for homing of T cells to lymphoid organs. Moreover, although we have not conducted a systematic analysis of adhesion responses in HS1-deficient T cells, we have never detected a requirement for HS1 in T cell adhesion to APCs (not shown).

Homing of naïve T cells to lymph nodes depends on CD62L (L-selectin), the chemokine receptors CCR7 and possibly CXCR4, and LFA-1 ($\alpha_L\beta_2$) and $\alpha_4\beta_7$ integrins (Bradley et al., 1994; Forster et al., 2008; Marelli-Berg et al., 2008). The signaling pathways downstream of chemokine receptors are poorly understood, although CXCL12 binding to CXCR4 induces tyrosine phosphorylation of cortactin (Luo et al., 2006).

It is therefore surprising that HS1 does not appear to be required for naïve T cell migration through lymphoid tissues. In addition to using NK cells, the Cooper lab studies were conducted *in vitro*, which contrasts with the *in vivo* studies presented here. Differences in cell type and experimental methodology, as well as the developmental stage of the cells used, may account for some of these discrepancies. Effector T cells use a different set of chemokine receptors to enter sites of inflammation (Marelli-Berg et al., 2008), and HS1 may function specifically in this context. It will therefore be important to test the role of HS1 in migration to sites of inflammation.

How is HS1 function regulated downstream of TCR engagement?

One of the first identified features of HS1 was its prominent tyrosine phosphorylation upon antigen receptor engagement (Hata et al., 1994; Takemoto et al., 1995; Yamanashi et al., 1993). We have shown that HS1 is phosphorylated in Jurkat cells at Tyr 378 and Tyr 397, most likely by ZAP-70 (Chapter 2 and (Gomez et al., 2006)). Mutation of these sites prevents Itk-dependent recruitment of HS1 to the IS and prevents HS1-dependent downstream signaling (Chapters 2 and 3). Because these tyrosines mediate binding to a host of signaling molecules, it seems unlikely that defective Itk-dependent IS recruitment is the sole cause of the signaling defects in cells expressing a variant of HS1 with Tyr→Phe mutations at these sites.

High-resolution video microscopy studies have demonstrated that signaling molecules are rapidly recruited to microclusters at sites of TCR engagement (Bunnell et al., 2002; Bunnell et al., 2006; Yokosuka et al., 2005). The cell spreading process that accompanies T cell activation, as well assembly of these signaling microclusters, are actin-dependent processes (Bunnell et al., 2001; Campi et al., 2005). WASp has been shown to localize initially to signaling microclusters, then to actin-rich sites (Barda-Saad et al., 2005). It seems likely that HS1 would follow a similar pattern, being initially recruited to signaling microclusters where it would undergo ZAP-70-dependent tyrosine phosphorylation. It would then redistribute to the periphery. Although we can detect some puncta suggestive of signaling microclusters at very early time-points, our data suggest that HS1 is primarily situated at the cell periphery in a pattern similar to that of

actin (Chapter 2). It is possible that HS1 is transiently recruited to these clusters before rapid redistribution, and that we have not detected these events in our experiments.

How does actin binding of HS1 influence its function?

Disease-associated variants provide particularly appealing tools to probe protein function, in that they are demonstrably physiologically relevant. A striking example is the discovery of mutations in WASp as the cause of Wiskott-Aldrich syndrome (Derry et al., 1994). Mutations in HS1, however, appear instead to contribute to a genetically complex disease, SLE. Although the individual contributions of the disease-associated variants, $HS1^{HTH2.5}$ or $HS1^{EP8}$, to SLE are likely to be small, and HS1 must interact with other risk factors to cause disease, these variants identify physiologically important features of these proteins. In this instance, the loss of an actin-binding repeat has provided us with a tool to assess the importance of actin binding in the regulation and function of HS1.

In preliminary studies, we have compared the function of a disease-associated actin-binding mutant, $HS1^{HTH2.5}$, with the more common variant, $HS1^{EP6}$. In a B cell AICD assay, $HS1^{HTH2.5}$ signals more efficiently than $HS1^{EP6}$ (Sawabe et al., 2003). Efficient F-actin binding by HS1 is required for efficient activation Arp2/3 complex and intact actin responses at the IS (Chapter 2). It is not clear from these studies whether the primary effect is on Arp2/3 complex activation, on the stability of actin branches, or is a combination of both. Moreover, the observed defect may reflect changes in the localization of HS1. We have not examined the effect of the HTH2.5 mutation on HS1

localization. However, cortactin requires the presence of the fourth HTH repeat for both actin binding and localization to the cell cortex (Weed et al., 2000), so it is anticipated that $HS1^{HTH2.5}$ will exhibit defects in localization to the IS. Proper interpretation of these data will require analysis of the half-life of branched actin filaments *in vitro* and kinetic analyses of actin polymerization and HS1 recruitment to the IS. These studies are currently underway. As HS1 deficiency also results in defects in Ca^{2+} flux and IL2 production, it will be interesting to examine the role of HS1 actin binding on TCR signaling.

Mutations in the HTH repeat region may not only directly affect actin binding, but may also have larger-scale effects on the structure of HS1. A structure for cortactin has been proposed in which the SH3 domain lies close to the HTH repeat region (Cowieson et al., 2008). If this structure also applies to HS1 and represents a closed, inactive form of the molecule, loss of one HTH repeat may alter the overall structure to a more open structure. In this model, the C-terminus, including Tyr 378 and 397 as well as the SH3 domain, is more exposed to potential binding partners. $HS1^{HTH2.5}$ was originally discovered in a SLE patient (Sawabe et al., 2003). It has not been determined how this mutant influences disease, or even in which cell type it is relevant. However, if this mutation alters the structure of HS1 as proposed, then signaling might occur more efficiently.

An allelic variant of HS1 containing eight glutamic acid-proline repeats, $HS1^{EP8}$, (Figure 1.4) instead of the more common six is more common in SLE patients than in healthy controls; this variant has been shown to have functional consequences for BCR

signaling that are similar to the HTH2.5 variant (Otsuka et al., 2004). These repeats lie four amino acids N-terminal to Tyr 378, a known phosphorylation site. Moreover, HS1 can be serine/threonine phosphorylated (Ruzzene et al., 2000) and contains a consensus MAP kinase recognition site at Thr 352 (Takemoto et al., 1995). MAP kinase-mediated phosphorylation of cortactin at Ser 405/418 can potentiate its ability to activate Arp2/3 complex, implying that this phosphorylation event induces a conformational change (Martinez-Quiles et al., 2004). The EP8 insertion may alter the structure of HS1 towards a more open conformation, due to either the negative charge of the insertion, which may be phospho-memetic, or to a simple increase in the overall length.

How does HS1 integrate with other components of the T cell actin regulatory machinery?

HS1 is required for to stabilize F-actin at the immunological synapse (Chapter 2). This results in the formation of unstable lamellipodial protrusions, and may explain the defects observed in Vav1 (Chapter 2) localization and PLCγ1 microcluster dynamics in HS1-deficient cells (Chapter 3). Although HS1 is capable of stimulating Arp2/3 complex-mediated actin branching, its activity in this regard is weaker than that of cortactin and the WASp VCA fragment (Uruno et al., 2001; Uruno et al., 2003b). Instead, HS1 extends the half-life of existing branched actin filaments, in agreement with apparent instability of F-actin structures that we have observed in T cells.

Itk is also required for actin responses in T cells and for Vav1 localization at the IS (Dombroski et al., 2005; Grasis et al., 2003; Labno et al., 2003). We now find that the

spreading defects in Itk-deficient cells resemble those of HS1-deficient cells, and that Itk is required for recruitment of HS1 to the IS (Chapter 3). Because Itk lacks a region that can directly interact with the actin cytoskeleton, the mechanism by which it regulates actin dynamics has been unclear. These data now establish HS1 as an effector of Itk in regulating actin dynamics. Itk is recruited to the plasma membrane via an interaction between its PH domain and PtIns(3,4,5)P_3 (August et al., 1997). In this model (Figure 5.1), TCR engagement leads to HS1 phosphorylation and binding to the SH2 domain of Itk. Localization at the IS allows HS1 to cross-link existing branched actin filaments, as well as to participate in the activation of Arp2/3 complex either through its own activity, or through interaction with other NPFs such as WASp. This also allows for the stabilization of Vav1 at the IS, where it can activate Cdc42 and Rac. This would in turn generate localized pools of Cdc42-GTP and Rac-GTP, which can activate WASp (Kim et al., 2000) or recruit WAVE2 (Steffen et al., 2004), respectively. Our laboratory has previously shown that Itk and Slp76 are required for the recruitment and localized activation of Vav1 (Dombroski et al., 2005; Labno et al., 2003; Zeng et al., 2003). Our data now establish HS1 as an additional player in this complex. Recruitment of Vav1 to the IS depends on the coordinate activity of these molecules. This is a complex process, and each protein to some extent probably plays a unique role. HS1 mostly likely functions to stabilize Vav1 and other signaling molecules such as PLCγ1, discussed below, at the IS by linking signaling complexes to the actin cytoskeleton. In the absence of Itk, HS1 is not recruited to the IS and to signaling complexes, and therefore cannot carry out this function. This would result in a loss of localized WASp activity,

potentially leading to more severe defects. Neither HS1 nor WASp is absolutely required for actin polymerization at the IS (see Chapter 2 and (Gomez et al., 2006) for HS1, and (Cannon and Burkhardt, 2004; Nolz et al., 2006) for WASp). In this respect, it would be interesting to test actin responses in cells lacking HS1 and WASp to probe for a compound phenotype. It is worth noting that WAVE2-deficient cells exhibit actin defects much more severe than cells lacking HS1, WASp, or Itk (Nolz et al., 2006). Therefore, it is likely that WAVE2 functions either independently of the pathways described here, or that other proteins, perhaps other Vav isoforms, can compensate for perturbations in this pathway.

Coronins are a set of poorly-understood F-actin-binding proteins that appear to function by inhibiting Arp2/3 complex-dependent nucleation (Humphries et al., 2002). Coronin1 deficiency results in increased F-actin content in resting as well as activated T cells (Foger et al., 2006; Mueller et al., 2008; Mugnier et al., 2008). In particular, upon APC encounter, the duration as well as the amount of F-actin at the IS in Coronin-deficient cells is increased. This is almost the opposite phenotype of HS1-deficient cells. It has been proposed that Coronin1 and cortactin antagonize one another in regulating lamellipodial actin dynamics (Cai et al., 2008). Loss of both proteins results in comparatively normal actin dynamics in HEK (human embryonic kidney) 293 cells. These two proteins localize at the leading edge, although cortactin lies somewhat farther from the periphery than cortactin. In T cells, HS1 and Coronin1 may exhibit a similar relationship. Although we have not attempted to colocalize these proteins in spreading T cells, the same may apply to HS1 and Coronin1. During the spreading process, HS1 is

responsible for stabilizing existing branched actin filaments at the leading edge. Coronin1, localized more towards the center, might disassemble branches, recycling actin monomers and Arp2/3 complex. Working together, these two proteins might regulate actin dynamics by controlling branch assembly and disassembly at specific locations within the cell.

What is the role of actin in Ca^{2+} signaling?

In Chapter 2 and Chapter 3, I show that HS1 is an important component of the signaling pathway leading to Ca^{2+} responses downstream of TCR engagement. As of this writing, no studies have been published on the regulation of Ca^{2+} signaling by cortactin, the non-hematopoietic homolog of HS1. Ca^{2+} flux responses in T cells consist of two phases. In the initial phase, endoplasmic reticulum stores are depleted, leading to the second phase, entry of Ca^{2+} from the extracellular milieu. Store depletion is initiated through the action of PLCγ isoforms (predominantly PLCγ1 in T cells (Ting et al., 1992)), which generate $Ins(1,4,5)P_3$ and DAG by hydrolyzing the membrane lipid $PtIns(4,5)P_2$ (Majerus et al., 1986; Rhee, 2001; Singer et al., 1997).

There is a precedent for phosphorylation-independent regulation of PLCγ1 activity by actin regulatory proteins. T cells lacking Coronin1 have also been shown to exhibit defects in Ca^{2+} store release as a consequence of reduced $Ins(1,4,5)P_3$ production, yet these cells do not have gross defects in PLCγ1 phosphorylation at the activating tyrosines (Mueller et al., 2008). PLCγ1 microcluster dynamics have not been examined in Coronin1-deficient cells.

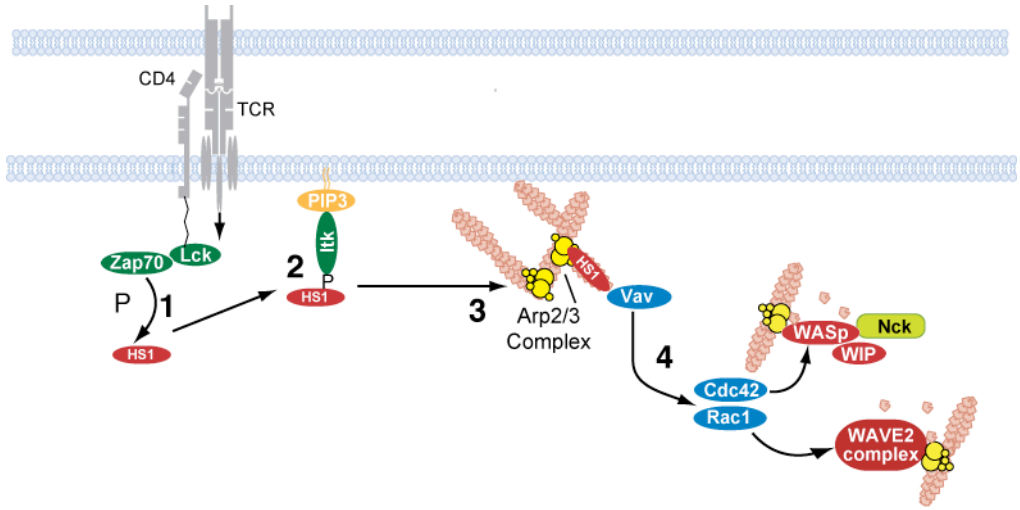


Figure 5.1: Itk-mediated recruitment of HS1 to the IS activates actin regulatory pathways.

TCR engagement leads to phosphorylation of HS1 (1). This allows Itk, which interacts with PtIns(3,4,5)P_3 (PIP3, in this figure) at the plasma membrane, to bind via its SH2 domain, enabling recruitment of HS1 to the IS (2). Once at the IS, HS1 can bind to and activate Arp2/3 complex, and stabilize branched actin filaments (3). HS1 also stabilizes Vav1 at the IS, leading to localized activation of Cdc42 and Rac1 and subsequent actin polymerization by WASp and WAVE2 (4).

It has been shown that Coronin1 and cortactin antagonize one another in regulating actin dynamics (Cai et al., 2008). If the same is true for Coronin1 and HS1, then the Ca^{2+} defects in HS1-deficient cells may be rescued by deletion of Coronin1 in those cells. Several groups have identified links between PLCγ isoforms and the actin cytoskeleton (Bar-Sagi et al., 1993; Dearden-Badet and Mouchiroud, 2005; Nojiri and Hoek, 2000; Payrastre et al., 1991; Yang et al., 1994). Together with the work presented here, these findings suggest that PLCγ1 function depends on intact actin dynamics. I propose that HS1 functions to stabilize PLCγ1 at the membrane, near its substrate, by tethering active PLCγ1 molecules to the actin cytoskeleton (Figure 5.2). This may be a general mechanism by which HS1 facilitates signaling. However, the mechanisms by which the actin cytoskeleton influences Ca^{2+} responses in T cells are complex, as discussed later in this section.

It is possible that PLCγ1 microclusters in HS1-deficient cells exhibit increased, albeit disordered mobility, due to loss of interactions with actin scaffolds. However, our experimental system, confocal microscopy imaging of cells expressing conventional YFP-tagged signaling molecules, does not permit single-molecule tracking, precluding direct testing of this hypothesis. Increased, disordered PLCγ1 mobility may prevent sufficiently stable interactions with its membrane-bound substrate to promote efficient $Ins(1,4,5)P_3$ production.

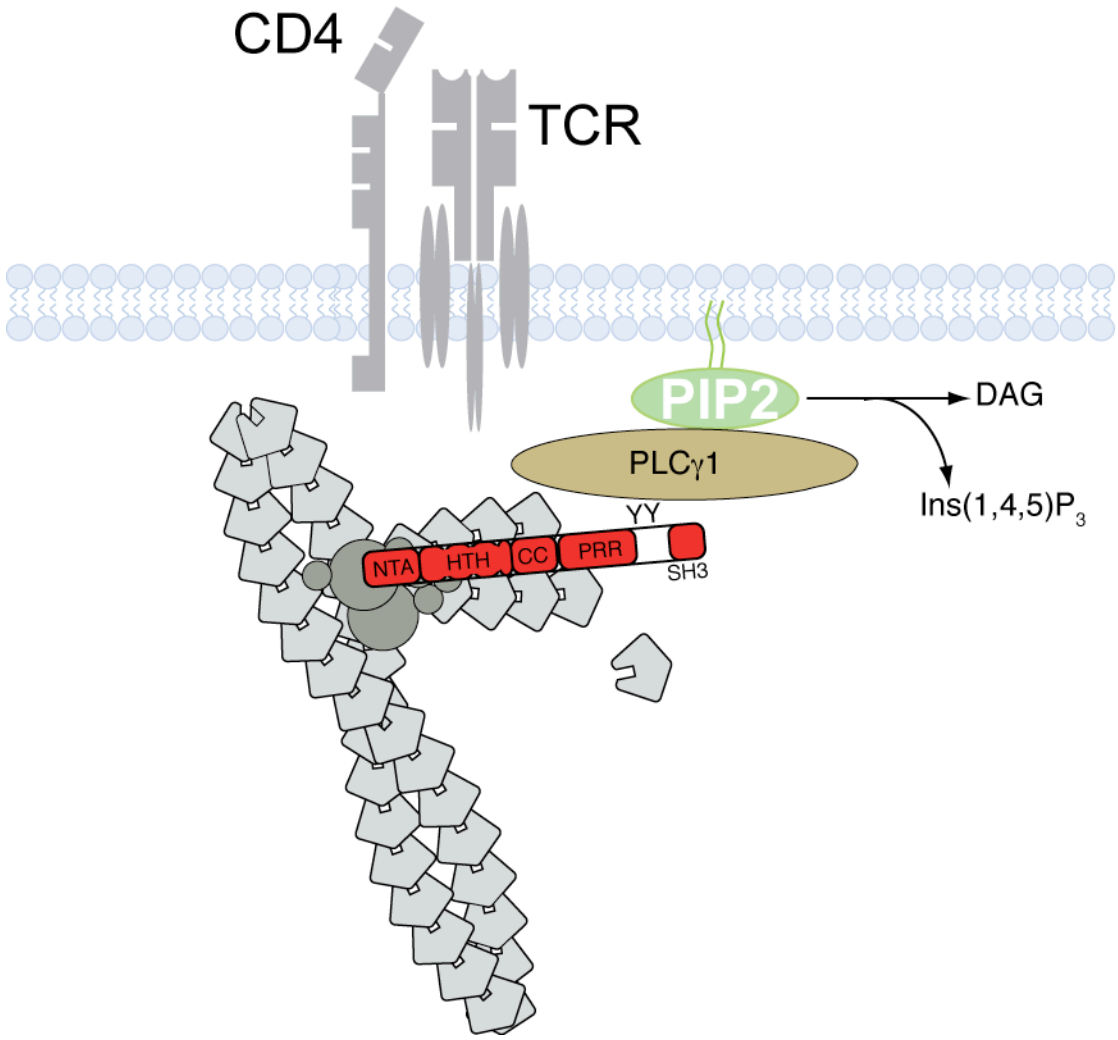


Figure 5.2: Model for HS1-mediated facilitation of PLCγ1 signaling

Phosphorylation of HS1 at Tyr 378 and Tyr 397 allows PLCγ1 to bind via its C-terminal SH2 domain. This interaction links PLCγ1 to actin scaffolds, restricting it near the membrane and facilitating interactions with the substrate, PtIns(4,5)P_2 (PIP2 in this figure).

In addition to acting as an adaptor protein linking PLCγ1 to the actin cytoskeleton, HS1 is also likely to be a component of larger scale signaling complexes. This is highlighted by our finding that phosphorylated HS1 can interact with a host of SH2 domain-containing proteins (Chapter 2). Loss of one component or uncoupling of the signalosome from the actin cytoskeleton may destabilize these macromolecular complexes (Bunnell et al., 2006), which may in turn impair PLCγ1 signaling. In support of this idea, low-dose Latrunculin B treatment of epithelial cells leads to severely reduced Ins(1,4,5)P_3 production (Suzuki et al., 2007). In this study, disruption of the actin cytoskeleton led to altered cell-surface receptor dynamics. This, in turn, reduced the specific, localized activation of PLCγ. There is evidence that the TCR, the relevant cell-surface receptor in T cells, interacts with the actin cytoskeleton (Rozdzial et al., 1995). We have not studied the effect of HS1 deficiency on TCR microcluster dynamics, but it may be that, in addition to regulating PLCγ1 microcluster dynamics, HS1 may affect TCR microclusters.

To more fully understand the roles that HS1 and other actin regulatory proteins play in microcluster dynamics would require a different experimental approach. Our current assay system, stimulation of cells on glass coverslips coated with anti-CD3, allows the formation of signaling microclusters, but in many instances they remain stationary rather than moving to a c-SMAC structure; a notable exception is Slp76 (Bunnell et al., 2002). This is probably because the activating ligand is immobilized on a glass substrate. The most physiologically relevant system would be to image

microcluster dynamics in T cells responding to APCs presenting peptides, which requires imaging over the Z-axis. However, due to the poor spatial and temporal resolution of three-dimensional images reconstructed from multiple sequentially imaged planes, it is necessary to employ a system in which the events of interest occur in the X-Y plane (Bunnell et al., 2003). The planar lipid bilayer system, notably employed by Dustin and coworkers, in which peptide-MHC complexes are incorporated and are free to diffuse laterally, is more amenable to the study of microcluster movement. In addition, this system employs a physiological ligand. However, it remains highly artificial in that it does not account for contributions from the cytoskeleton of the APC. Cytoskeletal rearrangements in dendritic cells are important for T cell activation (Al-Alwan et al., 2001a, b). The use of artificial "molecular mazes" in the lipid bilayer system to restrict the movement of peptide-MHC complexes (and other embedded molecules) may allow this system to more closely resemble a dendritic cell (Mossman et al., 2005). Nonetheless, it would be illuminating to examine the effect of HS1 deficiency on Slp76 microcluster dynamics (movement, lifetime, localization, and size), even with our current system. However, the lipid bilayer system with or without artificial barriers, particularly when employed with high-resolution TIRF (total internal reflection microscopy) imaging, may enable more elaborate and physiologically relevant analysis of the role of HS1 in microcluster assembly.

$Ins(1,4,5)P_3$ binding to its receptors on ER membranes induces store release. This is a highly cooperative process requiring the binding of three to four $Ins(1,4,5)P_3$ molecules (Marchant and Taylor, 1997; Meyer et al., 1988). This cooperativity has been

proposed to be a safeguard against spontaneous activation (Marchant and Taylor, 1997), but also implies that small, localized changes in PLCγ activity can lead to relatively large changes in the magnitude of store release.

Although it remains possible that HS1 influences store release independently of PLCγ1, this seems unlikely. We have shown that HS1 interacts with PLCγ1 in a phosphorylation-dependent manner (Chapter 2), and that HS1 deficiency results in abnormal PLCγ1 microcluster dynamics (Chapter 3). To further support the hypothesis that HS1 influences PLCγ1 activity would require direct measurement of PLCγ1-mediated Ins(1,4,5)P_3 production. This could be accomplished by measuring Ins(1,4,5)P_3 levels in lysates from WT or HS1$^{-/-}$ T cells. These assays are notoriously difficult, and although we could detect significant defects in Ins(1,4,5)P_3 production in HS1$^{-/-}$ T cells, these findings were not reproducible (not shown). Alternatively, a FRET (Förster resonance energy transfer)-based Ins(1,4,5)P_3 biosensor would permit assessment of Ins(1,4,5)P_3 levels in living cells (Remus et al., 2006). This system exploits the conformational change induced in the Ins(1,4,5)P_3 receptor upon ligand binding. These assays are appealing in that they enable analysis at the single-cell level over time, allowing one to gather information about variability within a population. Moreover, FRET-based assays can be read with flow cytometer, which would enable bulk measurement of Ins(1,4,5)P_3 production in complex populations, as has been done for protein phosphorylation (Krutzik et al., 2005a; Krutzik et al., 2005b). More relevant to the questions at hand, using a microscope would enable direct correlation of Ins(1,4,5)P_3

production with other parameters of T cell activation such as microcluster assembly and movement.

Other actin regulatory proteins also regulate Ca^{2+} flux responses downstream of TCR engagement, although they do so through a variety of mechanisms. The large GTPase Dynamin 2 is required for Ca^{2+} flux responses at the level of PLCγ1 phosphorylation (Gomez et al., 2005). Although total PLCγ1 phosphorylation is intact in Vav1-deficient Jurkat line J.Vav1 (Cao et al., 2002), $Vav1^{-/-}$ T cells also exhibit defects in PLCγ1 Tyr 783 phosphorylation (Reynolds et al., 2002). In contrast, WAVE2 is not required for PLCγ1 phosphorylation or release of Ca^{2+} from intracellular stores, but instead regulates Ca^{2+} entry from the extracellular environment (Nolz et al., 2006). $WASp^{-/-}$ T cells fail to sustain elevated levels of cytoplasmic Ca^{2+} (Zhang et al., 1999) and consequently have defects in NF-AT nuclear translocation (Cannon and Burkhardt, 2004), although the reason for this is unknown as of this writing. Interestingly, Jurkat cells suppressed for Arp2/3 complex expression, which respond to TCR engagement by generating actin-rich filopodia, have normal Ca^{2+} flux responses (Gomez et al., 2007). These varied findings indicate that regulation of Ca^{2+} by actin regulatory proteins such as HS1 is not merely through the organization of actin dynamics at the IS. Rather, it is likely that these proteins function as adaptors, linking signaling molecules, such as PLCγ1, to cytoskeletal scaffolds. Our finding that PLCγ1 localizes poorly to F-actin-rich fractions in $HS1^{-/-}$ cells supports this hypothesis (Chapter 3). Moreover, HS1-deficient Jurkat cells exhibit abnormal PLCγ1 microcluster dynamics (Chapter 3). Pharmacological disruption of actin dynamics has shown that microcluster assembly at

the p-SMAC, where active signaling is thought to occur, requires an intact actin cytoskeleton (Campi et al., 2005).

In some instances, disruption of the actin cytoskeleton can lead to increased Ca^{2+} flux responses or Ins(1,4,5)P_3 production (DeBell et al., 1992; Rivas et al., 2004; Valitutti et al., 1995). I have, under certain conditions, noted increased IL2 production from $HS1^{-/-}$ T cells relative to controls (not shown); even in these conditions, $HS1^{-/-}$ T cells exhibit a defect in actin polymerization at the IS. It is difficult to reconcile these findings with the model in which intact cytoskeletal dynamics are important for signaling microcluster assembly. However, this may reflect the fact that the actin cytoskeleton is also important for movement of existing microclusters from the p-SMAC to the c-SMAC, where signal downregulation occurs (Varma et al., 2006).

Using our conjugate assay system, we have shown that HS1 is required for the stability of F-actin and of two signaling molecules, Vav1 and PLCγ1, at the immunological synapse (Chapter 2 and Chapter 3). However, gross IS localization of these molecules is normal at early time points, during which the bulk of TCR-proximal signaling occurs. TCR-proximal signaling must proceed for hours for productive T cell activation to occur. Interruption of TCR engagement, even hours after APC encounter, impairs productive T cell activation (Huppa et al., 2003). Similarly, pharmacological inhibition of ZAP-70 kinase activity blocks Ca^{2+} signaling, even when the inhibitor is introduced after signaling has been initiated (Levin et al., 2008). Based on these findings, it is likely that the effects of HS1 on long-term Vav1 and PLCγ1 localization to the IS are physiologically important, even though these signaling molecules are associated with

very early TCR signaling events. I have discussed above a model whereby HS1 functions to facilitate Ca2+ signaling by tethering PLCγ1 molecules to the actin cytoskeleton (Figure 5.2). This is likely to be a general mechanism by which HS1 interacts with and regulates other components of the T cell signaling machinery.

Is there a role for HS1 in other signaling events?

PLCγ activity leads to the production of diacylglycerol in addition to Ins(1,4,5)P_3. DAG activates the Erk signaling cascade, which in turn activates the AP-1 transcription factor, a component of the *IL2* promoter (Smith-Garvin et al., 2009; Zhang and Dong, 2005). Although HS1 appears to be required for PLCγ1 activity, we have thus far found no defects in Erk phosphorylation or in AP-1 transcriptional activity in a luciferase reporter assay. The reason for this discrepancy is unknown.

HS1, through its proline-rich region, constitutively interacts with the SH3 domain of Lck (Takemoto et al., 1995). In addition, phosphorylation of HS1 creates binding sites for the SH2 domain of Lck (Chapter 2 and (Gomez et al., 2006)). As discussed in Chapter 1, this may represent a mechanism that allows Lck to phosphorylate HS1 at other sites, such as Tyr 222, a site identified *in vitro* (Brunati et al., 1999) and in platelets (Brunati et al., 2005). Using mass spectrometry, we did not detect, but cannot rule out, phosphorylation at this site. Another implication for this interaction is that HS1 binding may regulate Lck kinase activity. Proteins that interact in this way with kinases have been shown to promote tyrosine kinase activity by stabilizing the open, active conformation of the enzyme. This has been proposed both for the interaction between

Lck and Unc119, a poorly understood T cell signaling molecule, (Gorska et al., 2004) and for the interaction between Itk and Slp76 (Bogin et al., 2007). Lck is a crucial signaling molecule that is positioned at the earliest stage of the TCR signaling cascade and is required for HS1 tyrosine phosphorylation (Chapter 2 and (Gomez et al., 2006)). HS1-deficiency results in much milder signaling defects than Lck deficiency (Palacios and Weiss, 2004), and many tyrosine phosphorylation events are grossly intact. Therefore, HS1 cannot be absolutely required for Lck function. Instead, if HS1 binding enhances Lck kinase activity, it may serve to promote long-term signaling. It would be interesting to test the ability of HS1 to regulate kinase activity using *in vitro* kinase assays with purified proteins as well as with Lck immunoprecipitated from WT or HS1-deficient T cells. Unc119 has also been shown to be important for Lck trafficking, an actin-dependent process (Gorska et al., 2009). Disruption of actin dynamics through loss of HS1 may impair Lck trafficking. However, this would have to be a partial defect, because signaling is not completely blocked in HS1-deficient cells.

Concluding Comments

This work has demonstrated a role for HS1 in T cell function. We have shown that HS1 contains two tyrosines that are phosphorylated upon TCR engagement and mediate interactions with several molecules involved in TCR signaling. We showed that the interaction via these tyrosines with Itk is required for HS1 recruitment to the IS, where HS1 can stabilize actin filaments. These sites mediate an interaction with Vav1, and stabilize it at the IS, which may represent an additional mechanism by which HS1

regulates cytoskeletal dynamics. We showed that HS1 is required for release of Ca^{2+} from intracellular stores, likely due to a regulation of PLCγ1 dynamics by HS1. This study represents one of the first analyses of the regulation of signaling microclusters by a specific actin regulatory protein. Our studies of the HS1 knockout mouse demonstrated a role for HS1 in IFNγ production by $CD4^+$ T cells. However, it was surprising to find that several aspects of T cell function, including CTL-mediated killing and cytokine production, TCR endocytosis, and migration, were unaffected by the loss of HS1. Several open questions remain, as described in this chapter.

Studies using actin depolymerizing agents have demonstrated that intact actin dynamics are required for T cell function. In spite of this, this work has shown that HS1 deficiency does not result in severe signaling or functional defects. It seems likely that at least part of the reason for this is that other actin regulatory proteins, even non-homologous ones such as WASp, may play compensatory roles. However, it is also likely that each of these proteins plays selective roles within the context of T cell signaling. The challenge now is to probe for functional interactions among these molecules.

In vivo studies may ultimately provide insight into a potential role for HS1 in human disease. Many human diseases are genetically complex, involving contributions from multiple genetic polymorphisms that individually may be benign. A notable example of this is systemic lupus erythematosus (Moser et al., 2009). Already, two studies have linked polymorphisms in HS1 with SLE (Otsuka et al., 2004; Sawabe et al., 2003). Interestingly, a recent study has linked Coronin1 deficiency to SLE resistance in

mice (Haraldsson et al., 2008). Although the mechanism of action of HS1 in this disease is unknown, these studies establish a role for HS1 in disease. A more comprehensive genetic screen of patient samples (SLE and others) is likely to uncover additional associations. Ultimately, detailed study of disease-associated polymorphisms may require generation of "knock-in" mouse models expressing the genetic change in question.

Chapter 6: Materials and Methods

Reagents and Antibodies

All reagents are from Sigma unless otherwise specified. Antibodies against PLCγ1, Vav1, Lck and ZAP-70 have been previously described (Billadeau et al., 2000; Karnitz et al., 1992; Ting et al., 1992; Williams et al., 1998). Anti-human HS1, anti-mouse HS1, and anti-WIP were obtained by immunization of rabbits with a GST-fusion protein containing AA330-407 of human HS1, a GST-fusion protein containing AA226-351 of mouse HS1, or a KLH-conjugated synthetic peptide corresponding to AA468-494 of human WIP (Cocalico Biologicals). Rabbit anti-PLCγ1 phosphorylated at Y783 was from Cell Signaling Technologies. Mouse anti-PLCγ1 was from ECM Biosciences. Rabbit anti-WASp, mouse anti-Itk (clone 2F12) and anti-phosphotyrosine (4G10) were from Millipore. Anti-GST, anti-actin (C-2) and anti-Lamin B (M-20) were from Santa Cruz. Anti-GAPDH was from Calbiochem. Anti-MBP was from Immunology Consultants Laboratory. The anti-human CD3 (OKT3) was from the Children's Hospital of Philadelphia Pharmacy; anti-TCR (C305) was a gift from Dr. G. Koretzky (University of Pennsylvania). Antibodies against mouse CD3e (2C11) and CD28 (PV-1) were from the University of Chicago monoclonal facility. Anti-mouse CD3ε (500.A2) and mouse anti-human HS1 were from BD Pharmingen. Antibodies against Thy 1 (AT83.A) and CD24 (J11D) were gifts from Dr. A. Sperling (University of Chicago). Anti-CD3-PE, anti-CD4-allophycocyanin, anti-CD8-Pe/Cy7 (CD28.2), anti-CD25-PE, anti-CD69-PE, anti-CD44-FITC, anti-CD62L-PE, and anti-FoxP3-AlexaFluor488 were from Biolegend.

Goat anti-mouse IgG-IRDye800 was from Rockland Immunochemicals and goat anti-rabbit IgG Alexa Fluor 680 was from Molecular Probes. Goat anti-mouse IgG_1 Alexa Fluor 555 and goat anti-mouse IgG Alexa Fluor 594 were from Molecular Probes and donkey anti-mouse IgG Cy3 was from Jackson Immunoresearch.

Plasmids

Human HS1 was amplified from a cDNA library and mutated to generate the Y397F, Y378/397F, and shRNA-resistant mutants using the Quik Change™ kit from Stratagene (see Table 7.1). The IL2p.luc, NFκB.luc, and NF-AT.luc reporter constructs have been described (Cao et al., 2002).

The shRNA vectors pFRT-H1P and pCMS3.eGFP.H1p have been described (Gomez et al., 2005; Trushin et al., 2003). pCMS4.eGFP.H1p, derived from pCMS3.eGFP.H1p, contains an additional CMV promoter for driving the expression of shRNA-resistant proteins for reconstitution studies. shHS1b and shHS1f targeting sequences are in Table 7.1 along with the control shHS1mut sequence. The shRNA vector against Vav1 has been described (Zakaria et al., 2004). cDNA for human HS1 was tagged with the bright YFP variant Venus (Nagai et al., 2002) and cloned into the MSCV2.1 retroviral expression vector.

The Itk-myc expression constructs were gifts of Dr. L. Berg, University of Massachusetts. The shRNA vectors pFRT.H1p and pCMS3.eGFP.H1p, and the shHS1f targeting sequence have been described (Gomez et al., 2005; Gomez et al., 2006; Trushin

et al., 2003). siRNA duplexes against human Itk (Dombroski et al., 2005) were synthesized by Qiagen. For some studies, Itk suppression sequences were cloned into pFRT.H1p and pCMS3.eGFP.H1p as previously described (Gomez et al., 2005; Trushin et al., 2003). The following targeting sequences were used: shItkc: GAAGAAACGAGGAATAATA and shItke: GCACTATCCGATCCTCATC. All results were confirmed using both targeting constructs; results with only one construct are shown for simplicity. Electroporation was done using an ECM 830 square wave electroporator (BTX) in 500 μl antibiotic-free RPMI using a 310 V, 10ms pulse. Cells were used 24 hours after transfection with 40 μg Itk-myc expression constructs or 1.5 μg siRNA duplexes, or 72 hours after transfection with 40 μg shRNA vectors. In each case, suppression efficiency in the bulk population of cells was at least 60%.

Table 7.1: PCR primers and shRNA targeting sequences

Name	Sequence	Used for
5' HS1	5'-CTTGCACAGGTGAAGCTTTGGAAGTCTGTAGTGGGC-3'	PCR of HS1 cDNA[a]
3' HS1	5'-TTGCAGTAGACAGCGGCCGCTAGTCACTCCAGGAGCTTGACATAATTTGC-3'	PCR of HS1 cDNA[a]
shHS1b resistant mt	5'-GCAGCTCTGGGATATGAtTAtAAaGGAGAGACGGAGAAA-3'	Mutagenesis of HS1 cDNA making it resistant to shHS1b targeting[b]
shHS1b	5'-GACTACAAGGGAGAGACGGAG-3'	shHS1b targeting sequence
shHS1f	5'-GAACCAGAGGGGGACTATG-3'	shHS1f targeting sequence
shHS1bmut	5'-GACTAgAAGGcAcAGAgGGAG-3'	shHS1bmut non-targeting mutant sequence[b]
shItkc	GAAGAAACGAGGAATAATA	shItkc targeting sequence
shItke	GCACTATCCGATCCTCATC	shItke targeting sequence

[a] Underlining in these oligos indicates unique restriction sites used for subcloning the amplified HS1 cDNA.

[b] Lower case nucleotides indicate point mutations introduced into the primary sequence of HS1 cDNA or shHS1b.

Cell culture

All tissue culture reagents were from Invitrogen. Jurkat-E6, JCaM-1, P116, and primary human peripheral blood $CD4^+$ T cells, NALM6 B cells, Raji B cells, and EBV B cells were grown in RPMI-1640 supplemented with 5% FBS, 5% newborn calf serum, 25 mM HEPES, and 4mM glutamine. A Jurkat-E6 T cell line stably expressing GFP-actin was generated using the pEGFP-actin vector (Clontech). These cells express GFP-actin at ~37% of endogenous levels. Jurkat T cells stably expressing PLCγ1-eYFP ((Braiman et al., 2006), gift of Dr. L. Samelson, NIH) were maintained in RPMI containing 10% FBS, penicillin/streptomycin, glutamine, and 1 mg/ml G418. P815 mouse mastocytoma cells and the mouse B cell line CH27 were cultured in Glutamax DMEM (Gibco), with 5% FBS, non-essential amino acids, β-mercaptoethanol, penicillin and streptomycin, and 25 mM HEPES. DO11.10 T cell blasts were cultured as described (McKean et al., 2001). Murine $CD4^+$ T cells were isolated from lymph nodes and spleens by negative selection using a mixture of anti-MHC class II (M5/114.15.2) and anti-CD8 (2.43) followed by magnetic bead-conjugated goat anti-rat Ig (Qiagen). Where specified, total T cells were isolated using anti-MHC II alone. T cell blasts from WT or $HS1^{-/-}$ C57Bl/6 mice were prepared from $CD4^+$ T cells by stimulation on plates coated with anti-CD3 (2c11) and anti-CD28 (PV1) for 3 days, followed by 3-8 days of resting culture. Cells were maintained using DMEM supplemented with 10% FBS, non-essential amino acids, penicillin, streptomycin, HEPES and β-Mercaptoethanol (Sigma), supplemented with 50

U/ml rhIL2 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health; human rIL2 from M. Gately, Hoffmann-LaRoche, Nutley, NJ).

Mice

$HS1^{-/-}$ mice on the C57Bl/6J background have been previously described (Taniuchi et al., 1995) and were a gift of Dr. D. Rawlings (University of Washington). WT C57Bl/6 mice were from Jackson Laboratories. To generate T cells specific for moth cytochrome C 88-103 (MCC_{88-103}) presented on $I\text{-}E^k$, $HS1^{-/-}$ mice were crossed to AND TCR transgenic mice (Jackson Labs) (Kaye et al., 1989; Kaye et al., 1992; Vasquez et al., 1992), and maintained as heterozygotes for the AND transgene. C57Bl/6J mice were obtained from Jackson Laboratories. All mice were housed under pathogen-free conditions in the Children's Hospital of Philadelphia animal facility. All studies involving animals were reviewed and approved by the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee.

Isolation of primary T cells and primary APCs

T cells were prepared from spleens and lymph nodes by negative selection using either complement lysis (Chapter 2) or magnetic beads (Qiagen Biomag Goat anti-Rat) (Chapter 3 and Chapter 4. Primary APCs were prepared from wild type splenocytes by complement enrichment using anti-Thy1 (AT83.A), yielding less than 1% $CD3^+$ cells. Single cell suspensions were depleted of erythrocytes using Ack lysis buffer (150 mM

NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) and incubated with hybridoma supernatants for 30-60 minutes. Anti-CD24 (clone J11d), a kind gift of Dr. Anne Sperling, was used for complement lysis, and anti-MHCII (clone M5/114), anti-CD8.2 (2.43), and anti-CD4 (GK1.5) were used for magnetic bead isolation. For complement lysis, cells were incubated with rabbit complement H2 (Pel-Freez) for 30 minutes at room temperature with shaking then washed in DMEM. Dead cells were removed by centrifugation at 2000 RPM for 20 minutes over a Histopaque (Sigma) gradient. For magnetic bead isolation, cells were collected by centrifugation and incubated with Biomag Goat anti-rat IgG beads for 15 minutes at 4ºC. Bead-bound cells were removed with a Dynal magnet. Typical purity as assessed by flow cytometry staining was > 85%.

Preparation of T cell blasts

$CD4^+$ T cells from spleens and lymph nodes of WT or $HS1^{-/-}$ mice were activated on 6-well plates coated with (clone 145-2c11) and anti-CD28 (clone PV-1) for three days in the presence of 50 U/ml exogenous rhIL2 (recombinant human IL2). Blasting cells were transferred to fresh, uncoated 6-well plates and cultured in the presence of exogenous rhIL2 (100 U/ml) for three to eight additional days.

Transfection of Jurkat cells by electroporation

Jurkat cells growing in log phase were collected by centrifugation and resuspended at $2x10^7$ cells/ml in RPMI-1640 supplemented with 5% FBS, 5% newborn calf serum, 25 mM HEPES, and 4mM glutamine. 500 µl of cells were transferred to a 4 mm gap

cuvette, mixed with DNA, and electroporated at 310 V, 1 ms pulse using a BTX ECM 830 electroporator (Harvard Apparatus). For shRNA experiments, cells were allowed to recover for 48-96 hours prior to use. For overexpression experiments, cells were used 16-24 hours after transfection.

Luciferase reporter assays

Transient expression and/or suppression and luciferase reporter assays in Jurkat were done as previously described (Cao et al., 2002; Nolz et al., 2006). For cell-cell stimulation in reporter assays, live NALM6 B cells ($1x10^6$) and 0.5μg/ml Staphylococcal Enterotoxin E (SEE, Toxin Technologies) were added to each well. For cell-cell stimulation time courses, Jurkat cells were activated with fixed, SEE-pulsed NALM6 or RAJI B cells as described (Gomez et al., 2005).

Retroviral transduction of primary mouse T cells

CD4+ T cells were stimulated for three days as described under "Preparation of T cell blasts." Cells were then transduced by resuspending in 1 ml retroviral supernatant containing 8 mg/ml polybrene and 50 U/ml rhIL2 and centrifugation at 823 x *g* in a 24 well plate for 2 hours at room temperature. Transduced cells were cultured in 24 well plates in media containing 50 U/ml rhIL2 for four to five days. Transduction efficiency was assessed by flow cytometry at 48 hours after transduction by staining with mouse anti-NGFR hybridoma supernatant (ME20.4) and PE-anti-mouse IgG (Biolegend) as described above.

Magnetic sorting of retrovirally transduced cells

T cells were resuspended in MACS buffer (PBS containing 0.5% BSA, 2mM EDTA) supplemented with 25% anti-NGFR hybridoma supernatant and incubated at 4ºC for 15 minutes. Cells were then collected, washed twice in MACS buffer, and incubated with anti mouse IgG MACS beads (Miltenyi Biotec) at 4ºC for 15 minutes. Bead-bound cells were positively selected using MACS LS columns, and cultured for overnight prior to use.

Flow cytometry

For cell-surface staining, samples were washed in FACS buffer (5% FBS, 2 mM EDTA, and 0.02% NaN_3 in PBS), resuspended in 100 μl of FACS buffer, and incubated at 4ºC with appropriate antibodies for 20 minutes. Samples were washed in FACS buffer, incubated with secondary antibodies as necessary for 15-20 minutes, and washed twice. Samples were analyzed immediately or fixed in 0.5% paraformaldehyde. For intracellular cytokine staining, cells were fixed at room temperature for 10 minutes with 3% paraformaldehyde, then washed once in FACS buffer and once in permeabilization buffer (1% FBS, 0.1% saponin in PBS). Samples were incubated in 100 μl permeabilization buffer containing anti-IFNγ and anti-IL4 for 30 minutes, followed by two washes in FACS buffer and immediate analysis. All flow cytometry data acquisition was conducted on a FACSCalibur (BD Biocsciences) and analyzed using FlowJo 6.0 (TreeStar).

Helper T cell differentiation

$CD4^+$ T cells ($2.5x10^5$/well) from WT or $HS1^{-/-}$ mice were stimulated on 24-well plates coated with 1 or 0.1 μg/ml anti-CD3 (145-2c11) and 1 μg/ml anti-CD28 (PV-1) for three days. Cells were then transferred to fresh plates and cultured in the presence of 50 U/ml rhIL2 for four days. Cells were assayed for cytokine production by stimulating with PMA (32 nM) and Ionomycin (500 nM) for two to three hours, then exposed to Brefeldin A (1 μg/ml) for two hours. Cells were washed in FACS buffer, and then stained as described above.

Generation of Jurkat cells stably expressing VenusHS1

Jurkat cells were transfected with MSCV-Venus-HS1 expression vector and allowed to recover for two days prior to selection in 1 mg/ml G418. After two weeks, a population of G418-resistant cells emerged. This culture was expanded and sorted on cells expressing low, medium, and high levels of Venus using a FACSVantage cell sorter (BD Biosciences). Sorted cells were then plated at one cell per well of a flat-bottom 96 well plate by limiting dilution. Two clones, 6c7 and 8g5, expressing approximately a 1:1 ratio of Venus-HS1:endogenous HS1 were selected for future use.

Cytotoxicity assays

CTL were generated *in vitro* by stimulating $CD8^+$ T cells from lymph nodes and spleens of WT or $HS1^{-/-}$ mice on the C57Bl/6 background ($H\text{-}2^b$) on plates coated with anti-CD3

and anti-CD28 for three days in the presence of 50 U/ml exogenous rhIL2. Cells were then transferred to fresh plates and cultured for two additional days prior to use. For cytotoxicity assays, effector CTL were plated in plated in 96-well round bottom plates at 3-fold serial dilutions, starting with 5x105 cells per well. Allogeneic target cells (P815 mouse mastocytoma cells, H-2^d) were loaded with ^{51}Cr, washed, and plated ($1x10^4$ cells/well). Cells were incubated at 37ºC for four to five hours. To control for spontaneous ^{51}Cr release, some wells received only target cells. To determine maximal release, some wells received only target cells, which were lysed with 1% NP40 (Sigma). Supernatants (100 μl) were harvested to 96-well Luma plates (PerkinElmer) and allowed to evaporate overnight. Plates were read on TopCount scintillation counter (PerkinElmer).

TCR internalization

TCR internalization studies were conducted according to (Cemerski et al., 2007). All manipulations were conducted in DMEM containing 1% FBS. $CD4^+$ T cells prepared by negative selection from WT or $HS1^{-/-}$ AND TCR transgenic mice. For peptide loading, CH27 B cells were incubated at 37ºC with 0.1-10 μM MCC_{88-103} peptide for four hours. CH27 cells without preloaded peptide served as controls. T cells and B cells were each washed and resuspended at $1x10^6$/ml. T cells and B cells were then mixed at a 1:1 ratio in V-bottom plates, $1x10^5$ of each cell type, and incubated for the indicated length of time. Conjugates were then harvested, washed in FACS buffer, and stained with anti-

CD4, anti-Vα11, and anti-Vβ3 for analysis by flow cytometry. Only $CD4^+$ events were analyzed to exclude CH27 cells not in conjugates.

Activation-induced cell death

AICD studies were conducted according to a protocol from Dr. Richard Siegel (NIH). $CD4^+$ T cell blasts (above) were used three days after removal from stimulatory plates. To induce AICD, cells were resuspended at $2x10^6$/ml in DMEM containing 50 U/ml rhIL2 and stimulated on flat-bottom 96-well plates coated with the indicated concentrations of anti-CD3 for eight hours. Cells were harvested and stained for 20 minutes with Annexin-V-AlexaFluor 647 (Molecular Probes) and 7-AAD (Molecular Probes) in Annexin binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Data were acquired on a FACSCalibur (BD Biosciences) and analyzed using FlowJo (TreeStar, Inc.). Percent specific cell death was calculated according to the following equation: $1 - \frac{\%\ Live_{treated}}{\%\ Live_{untreated}} \times 100$.

ELISA

For IL2 ELISA, total T cells ($2x10^5$ cells per well) were plated in a 96-well round-bottom TC plate (Costar) with 50,000 T-depleted splenocytes and SEB (Toxin Technologies). After incubation at 37ºC for 24h, plates were frozen at -80ºC. IL2 ELISAs were performed using the mouse IL2 Ready-SET-Go! ELISA kit (eBiosciences). For IFNγ ELISA, $CD4^+$ or $CD8^+$ T cells isolated from spleens and lymph nodes of WT or $HS1^{-/-}$ mice were stimulated at $1x10^6$ cells/ml on 96-well flat-bottom plates ($2x10^5$ cells per

well) coated with anti-CD3 (clone 145-2c11, 1.0 or 0.1 μg/ml) and anti-CD28 (clone PV-1, 1 μg/ml) for three days, after which plates were frozen at -80ºC. Supernatants were assayed for IFNγ content using the mouse IFNγ Ready-SET-Go! kit (eBioscience) according to the manufacturer's instructions.

Analysis of surface marker upregulation

Total T cells were stimulated as described for IL2 ELISA except that 1 μg/ml anti-CD3 (145-2c11) was used instead of SEB. After 24 hours, samples were stained and analyzed by flow cytometry as described above.

F-actin binding assay

Recombinant GST-tagged $HS1^{EP6}$ or $HS1^{HTH2.5}$ was produced in *Escherichia coli* and affinity purified on a glutathione resin (Amersham) and used in an Actin Binding Protein Spindown Assay Kit for non-muscle actin (Cytoskeleton, Inc.) according to the manufacturer's instructions. Briefly, 1 μM GST-HS1 was incubated with the indicated concentrations of F-actin for 30 minutes at room temperature in . Samples were then centrifuged at 150,000 x *g* for 90 minutes at room temperature. Supernatants were transferred to fresh tubes, and both supernatants and pellets were analyzed by coomassie staining or by western blotting for GST. BSA was used as a negative control, and α-actinin was used as a positive control.

Actin polymerization assay

In vitro actin polymerization was assessed using an actin polymerization kit (Cytoskeleton, Inc.), according to the manufacturer's instructions. Briefly, recombinant GST-tagged WASp VCA fragment (80 nM) or GST-tagged $HS1^{EP6}$ or $HS1^{HTH2.5}$ (100 mM) were incubated with 70 nM Arp2/3 complex in actin polymerization buffer (50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP) in a 96-well plate. Reactions were initiated by the addition of actin containing 20% pyrene labeled actin and monitored every 30 seconds using a fluorescence plate reader.

Immunofluorescence microscopy

Jurkat T cells were conjugated to Raji B cells at 37ºC as described previously (Cannon et al., 2001) and stained for F-actin using Alexa Fluor 647-phalloidin (Molecular Probes) and for HS1 followed by anti-mouse IgG Alexa Fluor 594 or anti-mouse IgG Cy3. For experiments involving $CD4^+$ T cells from AND transgenic mice, CH27 B cells were labeled with CMAC (Cell tracker blue, Molecular Probes) and incubated with 5 μM moth cytochrome C peptide ($MCC_{88\text{-}103}$). Equal numbers of T cells and B cells were centrifuged together, incubated at 37ºC for various times, and plated on poly-L-lysine-coated coverslips followed by fixation in 3% paraformaldehyde. Excess aldehyde groups were quenched by incubation in 0.5 mg/ml $NaBH_4$. Samples were blocked in TSGxx. Conjugates were stained for PLCγ1, Itk, or F-actin using Alexa Fluor 647-phalloidin followed by anti-mouse IgG_1 Alexa Fluor 555. Conjugates were analyzed on a Zeiss Axiovert 200M microscope equipped with a 63x planapo 1.4 NA objective. Images were collected using a Photometrics Coolsnap FX-HQ (Roper Scientific) camera and

deconvolution was performed using a constrained iterative algorithm (Slidebook v. 4.2, Intelligent Imaging Innovations). Alternatively, conjugates were imaged on a Zeiss Axiovert 200 equipped with a Perkin Elmer Ultraview ERS6 spinning disk confocal system and a 63x planapo 1.4 NA objective. Images were collected using an Orca ER camera (Hamamatsu) and analyzed using Volocity v. 5 (Improvision). For analysis, conjugates were identified at random as a blue B cell in contact with a T cell, and scored for the presence of a bright band of protein at the cell-cell contact site. All experiments were analyzed by an individual blinded to the experimental conditions. At least 50 conjugates were scored per condition in each experiment.

Live cell imaging

Eight well Lab-Tek II chambered coverglasses (Nalge Nunc) were cleaned with 70% ethanol containing 1 M HCl prior to incubation for 15 minutes with 0.1 mg/ml poly-L-lysine. Prior to use, coverglasses were coated with 10 μg/ml OKT-3 for 2 hours at 37ºC or overnight at 4ºC. Coverglasses were rinsed in PBS and covered with 400 μl RPMI without phenol red immediately prior to imaging and equilibrated to 37°C on the microscope stage within a Solent environmental chamber. Cultured cells were resuspended in phenol red-free RPMI at $2x10^6$/ml, and spreading was initiated by adding 5-10 μl of cell suspension to the coverglass chamber. Time-lapse images were collected at 63X using a Perkin-Elmer ERS6 Ultraview spinning disk confocal system. Stacks of 3-7 images were collected at 0.5 μm spacing every 3-5 seconds for approximately 6 minutes. GFP-actin dynamics were analyzed using Volocity Quantitation for area

measurements and Slidebook for radial variance as follows: Projections were created from the 4D datasets, masks created for each cell at each time point and the areas determined in pixels. For radial variance calculations, 64 radii were struck from the centroid of each mask, and the standard deviation of the radii was calculated for each cell at each time point. Video sequences from several cells were aligned based on initial contact with the coverslip and the average of the cell area and of the radial variance were calculated for the population of cells, at each time point. At least 40 cells were analyzed per condition. To assess PLCγ1 microcluster dynamics, maximum intensity projections were generated, and individual objects were identified based on pixel intensity and tracked using Volocity. Microcluster area at 90 seconds after contact with the coverslip for each individual size. At least 20 cells were analyzed per condition. Objects were pseudocolored based on time of appearance, relative to the total duration of each time-lapse sequence.

Ca^{2+} assays

For single cell Ca^{2+} measurements, freshly isolated $CD4^+$ T cells were loaded with the cell-permeant Ca^{2+} indicator Fura-2 AM (3.0 μM, Molecular Probes) for 15 min in normal bath solution (155 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM Hepes (pH 7.4)). Cell suspensions were placed into the recording chamber on an inverted fluorescence microscope (Nikon) and allowed to adhere to Poly-L-lysine-treated (100 μg/ml, Sigma) coverslips for 5 minutes. Extracellular Fura-2 AM was removed by perfusion with additional normal bath solution. Before stimulation, the

chamber was perfused with Ca^{2+} free bath solution containing 155 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$ and 0.5 mM EGTA, 10 mM glucose and 10 mM Hepes (pH = 7.4). Intracellular Ca^{2+} mobilization was initiated by addition of anti-CD3 (500.A2) in Ca^{2+}-free bath solution; consequently, the initial Ca^{2+} transient observed in Ca^{2+}-free solution is due to Ca^{2+} release from stores. The activation state of calcium entry channels was then examined by replacing the Ca^{2+}-free solution with normal (Ca^{2+}-containing) bath solution. Where indicated, 1 μM thapsigargin was added to block SERCA pump activity, thereby depleting intracellular Ca^{2+} stores. Fura-2 was alternately excited at 340 nm and 380 nm and fluorescence emission (plotted as the 340 nm/380 nm ratio) of individual cells was measured by digital imaging microscopy using Metafluor software (Molecular Devices).

Western blotting

Lysates were separated on Tris-Glycine SDS-PAGE gels, transferred to immobilon-FL (Millipore) or nitrocellulose (Bio-Rad) membranes, blocked in 3% non-fat dry milk in TBS and probed with primary antibodies in 3% milk in TBST or 3% BSA in TBST for phospho-specific antibodies. Blots were probed with goat anti-mouse IgG-IRDye800 or goat anti-rabbit IgG-Alexa Fluor 680 and imaged on a Licor Odyssey infrared fluorescence scanner.

GST Pulldowns

Fragments comprised of the SH3-SH2 domains (AA154-352) or the SH2 domain alone (aa 237-352) of human Itk were cloned into pGEX-KG and mutations to inactivate the SH3 (W208/209Y) and/or SH2 (R265A) domains were incorporated using standard molecular biology techniques. The SH3 domain of HS1 (AA415-486) was cloned into pGEX-KG and inactivating mutations (W455Y, W456Y) were incorporated. Recombinant GST fusion proteins were generated in bacteria and bound to glutathione resin (Amersham). Jurkat cells were harvested, resuspended in PBS with Ca^{2+} and Mg^{2+}, allowed to rest at 37ºC for 20 minutes, and treated with pervanadate (1:33 dilution) for 3-5 minutes or left untreated. Pervanadate was prepared by mixing equal parts Na_3VO_4 and 3% H_2O_2 and allowed to react at room temperature for 10 minutes. Cells were collected by centrifugation and lysed in NP40 lysis buffer (50 mM Tris-HCl pH 8, 1% NP40, 100 mM NaCl, 5 mM EDTA, 0.5mM $CaCl_2$, protease inhibitors, 1 mM Na_3VO_4, and 5 mM NaF). Lysates were clarified by centrifugation and exposed to glutathione resin-bound GST fusion proteins for 1-2 hours. Beads were washed three times in NP40 lysis buffer and analyzed by western blotting and Coomassie staining. Phosphorylated GST-HS1 fusion peptides (AA361-436) were made in the TKB1 *E. coli* (Stratagene). For *in vitro* binding assays, 30μg of p-GST fusion protein was bound to glutathione-agarose, washed, incubated with 2 μg of maltose binding protein (MBP)-fused Vav1-SH2 proteins, rotated at 4°C for 20 minutes, and then washed four times with lysis buffer.

Coimmunoprecipitations

Cultured $CD4^+$ T cell blasts were harvested and resuspended at $1x10^8$/ml in serum-free DMEM for antibody stimulation or in PBS with Ca^{2+} and Mg^{2+} for pervanadate treatment. Cells were allowed to rest for 30 minutes at 37ºC, then labeled on ice with 10 μg/ml biotinylated anti-CD3 (2c11) for 20 minutes. Signaling was initiated by the addition of 2.5 μg/ml streptavidin at 37ºC. At the indicated time points, cells were collected by centrifugation and lysed in NP40 lysis buffer. Postnuclear lysates from $4x10^7$ cells were incubated with anti-mouse HS1 bound to protein A-sepharose for two hours at 4ºC, washed in lysis buffer, eluted in 2x SDS-PAGE sample buffer, and analyzed by western blotting for HS1, Itk, and phosphotyrosine.

Preparation and phosphorylation of recombinant HS1

His-Tagged HS1 was expressed in *E. coli*. Following IPTG induction, bacteria were lysed with phosphate buffer (20mM $NaPO_4$, 1% NP40, 500mM NaCl, 20mM Imidazole, pH 7.2) with EDTA-free protease inhibitors (Roche). Lysates were clarified by centrifugation and loaded onto a HiTrap column (Amersham), pre-charged with 100mM $NiSO_4$ on an AKTA FPLC. The column was washed with phosphate buffer and eluted with a gradient to 500mM Imidazole. The appropriate fraction was polished over a HiTrap Q HP column and eluted with a gradient to 1M KCl. 20μg of recombinant HS1 was incubated with or without 0.5μg recombinant Syk (Upstate) in 50mM Tris pH7.5, 100μM $NaVO_4$, 0.1% β-mercaptoethanol, 5mM $MgCl_2$,100μM ATP for 10 min. at 30°C.

Mass Spectrometry

Recombinant HS1 or HS1 immunoprecipitated from Jurkat cells was resolved by SDS-PAGE, stained with Coomassie R-250 (Pierce), excised, and washed extensively with 50:50 (acetonitrile/25 mM ammonium bicarbonate, pH 8.0). Rehydrated gel pieces were infused with trypsin (Promega; 12.5ng/μl) or Glu-C (Roche; 20 ng/μl), at pH 8.0, and digested at 37°C for 18-24h. Reactions were stopped by addition of 5% TFA. Non-peptide impurities were removed using C_{18} or SCX ziptips (Millipore) and analysis was carried out in 0.1% phosphoric acid. MALDI-TOF spectra were acquired on a Ciphergen SELDI mass spectrometer in the linear mode. vMALDI Ion-Trap spectra were acquired on a Finnegan LTQ mass spectrometer. Following the MS scan, a data-dependent "Top 9" experiment was acquired such that 1 full MS spectrum was first acquired followed by 9 MS^2 spectra. For database searching we used Bioworks 3.2 employing the SEQUEST algorithm, against the SWISSPROT database. To confirm that phosphopeptides were present, a neutral loss-dependent MS^3 experiment was performed, and peaks that showed loss of 98 Daltons were subjected to further MS/MS.

Analysis of PLCγ1 phosphorylation and cytoskeletal association

Total T cells isolated from the spleen and lymph nodes of wild-type or $HS1^{-/-}$ C57Bl/6 mice were resuspended at $1x10^8$/ml in DMEM and rested at 37ºC for 30 minutes. Cells were stimulated by the addition of 5 μg/ml anti-CD3 (500.A2). Stimulation was stopped by transferring an aliquot of cells to a tube containing ice-cold PBS. Cells were collected by centrifugation, lysed in RIPA buffer (50 mM Tris pH 8, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, protease inhibitors, 5 mM NaF, 1 mM Na_3VO_4,

5 mM EDTA). Lysates were incubated on ice for 20 minutes, then clarified by centrifugation at 16,000 x *g*. Samples were analyzed by western blotting. For isolation of insoluble F-actin-rich fractions, cells were lysed in cytoskeletal stabilizing lysis buffer (80 mM PIPES pH6.9, 1% Triton X-100, 1 mM EGTA, 1 mM $MgCl_2$, protease inhibitors, 5 mM NaF, 1 mM Na_3VO_4), and centrifuged immediately at 5,000 x *g* for 5 minutes (Gatfield et al., 2005). The F-actin rich pellet was resuspended in high salt buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, protease inhibitors, 5 mM NaF, 1mM Na_3VO_4) and vortexed for 1 hour at 4ºC to extract proteins. Samples were centrifuged at 16,000 x *g* for 10 minutes, and supernatants were used as the insoluble fraction for western blot analysis. The amount of PLCγ1 or phospho-PLCγ1 in the pellets was quantified by normalizing to Lamin B intensity within each lane, then to the normalized value for unstimulated cells. Lamin B was used as a loading control because the insoluble fractions contain nuclei.

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