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Uncoupling Activation-Dependent HS1 Phosphorylation from Nuclear Factor of Activated T Cells Transcriptional Activation in Jurkat T Cells: Differential Signaling Through CD3 and the Costimulatory Receptors CD2 and CD28

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CD3, CD2, and CD28 are functionally distinct receptors on T lymphocytes. Engagement of any of these receptors induces the rapid tyrosine phosphorylation of a shared group of intracellular signaling proteins, including Vav, Chl, p85 phosphoinositide 3-kinase, and the Src family kinases Lck and Fyn. Ligation of CD3 also induces the tyrosine phosphorylation of HS1, a 75-kDa hematopoietic cell-specific intracellular signaling protein of unknown function. We have examined changes in HS1 phosphorylation after differential stimulation of CD3, CD2, and CD28 to elucidate its role in T cells and to further delineate the signaling pathways recruited by these receptors. Unlike ligation of CD3, stimulation with anti-CD28 mAb or CHO cells expressing the CD28 ligands CD80 or CD86 did not lead to tyrosine phosphorylation of HS1 in Jurkat T cells. Additionally, no tyrosine phosphorylation of HS1 was induced by mitogenic pairs of anti-CD2 mAbs capable of activating the transcription factor NFAT (nuclear factor of activated T cells). Costimulation through CD28 and/or CD2 did not modulate the CD3-dependent phosphorylation of HS1. In vivo studies indicated that CD3-induced HS1 phosphorylation was dependent upon both the Src family tyrosine kinase Lck and the tyrosine phosphatase CD45, did not require MEK1 kinase activity, and was regulated by protein kinase C activation. Thus, although CD3, CD28, and CD2 activate many of the same signaling molecules, they differed in their capacity to induce the tyrosine phosphorylation of HS1. Furthermore, activation-dependent tyrosine phosphorylation of HS1 was not required for NFAT transcriptional activation. The Journal of Immunology, 1998, 161: 4506–4512.

Resting T lymphocytes require at least two distinct signals for proliferation and differentiation into functionally active effector T cells. One of these signals can be provided by engagement of the Ag-specific TCR (TCR/CD3) complex. Engagement of the TCR/CD3 complex alone, without a second costimulatory signal, can lead to T cell unresponsiveness or cell death by apoptosis (1–3). Additional intracellular signals are triggered when the costimulatory receptors CD2 and CD28 bind to their respective ligands on the surface of APCs. Costimulation through CD28 or CD2 dramatically increases the production of several lymphokines, including IL-2 (3–5), through transcriptional up-regulation and stabilization of cytokine mRNA and can regulate the induction of T cell anergy (6). In contrast to TCR signal, engagement of CD28 can send a calcium-independent signal that is insensitive to the immunosuppressive drugs cyclosporin A and FK506, inhibitors of the intracellular serine/threonine phosphatase calcineurin (7, 8). Engagement of either CD3, CD28, or CD2 can trigger shared signaling pathways, including activation of phospholipase Cγ1; phosphoinositide 3-kinase (PI 3-kinase)4; Raf; and the cytoplasmic protein tyrosine kinases Lck, Fyn, and Bk (9–12). CD28 costimulation has been reported to enhance T cell survival following TCR stimulation by increasing the expression of Bcl-xL (13) and by inhibiting the induction of Fas ligand through distinct yet undefined pathways (14).

HS1 is a 75-kDa intracellular protein of uncharacterized function, expressed only in hematopoietic cells. It was originally cloned from a B cell cDNA library using a probe to the adenovirus 2 transcription factor E1A (15). HS1 has since been independently isolated on three separate occasions because of its pronounced tyrosine phosphorylation after engagement of the B cell Ag receptor (16) and the FceRI receptor on mast cells (17) and because of its binding to the Src homology 3 (SH3) domain of the cytoplasmic protein tyrosine kinase Lck (18). HS1 has several interesting structural features that suggest that it may be an important immunoregulatory protein. The amino-terminal region contains three copies of a 37 amino acid-repeating helix-turn-helix motif similar to that found in many DNA-binding proteins and in the tyrosine kinase substrate cortactin (19). The carboxyl-terminal region contains a possible nuclear localization signal, an SH3 domain, a proline-rich region, and two acidic α-helices that resemble the activating region of prokaryotic transcriptional activator proteins.

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4 Abbreviations used in this paper: PI 3-kinase, phosphoinositide 3-kinase; SH3, Src homology 3; NFAT, nuclear factor of activated T cells; PRC, protein kinase C; CHO, Chinese hamster ovary; PMA, phorbol 12-myristate 13-acetate; PVDF, poly(vinylidene difluoride).
HS1 is found in both cytosolic and nuclear cell fractions (16). Although recent studies suggest that HS1 may be involved in the apoptotic response to stimulation of the Ag receptor on B and immature T cells (20–23), the function and regulation of HS1 in lymphocytes remain to be established.

We investigated activation-dependent changes in the tyrosine phosphorylation of HS1 in Jurkat T cells in response to a diverse set of stimulation conditions. In contrast to CD3, engagement of the costimulatory receptors CD28 and CD2 did not induce the tyrosine phosphorylation of HS1. We found that T cell stimulation conditions (e.g., mitogenic pairs of anti-CD2 mAbs) that were capable of activating the nuclear factor of activated T cells (NFAT) did not induce activation-dependent HS1 tyrosine phosphorylation. The in vivo regulation of HS1 tyrosine phosphorylation by CD3 was then examined. CD3-induced HS1 phosphorylation was dependent upon both the Src family tyrosine kinase Lck and the tyrosine phosphatase CD45, did not require MEK1 kinase activity, and was regulated by protein kinase C (PKC) activation.

Materials and Methods

Cell lines and cell culture

The T cell leukemia cell line Jurkat clone J77 was kindly provided by K. Smith (Cornell University, NY, NY). The Jurkat subclones JCam1 (deficient in Lck tyrosine kinase activity) and J45.01 (deficient in the tyrosine phosphatase CD45) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Jurkat cells were routinely cultured at 37 °C in 5% CO2 in 10% RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% heat-inactivated FCS (Sigma, St. Louis, MO); 100 U/ml penicillin; 100 μg/ml streptomycin; 2 mM glutamine; 15 mM HEPES, pH 7.2; and 2 mM glutamine (Life Technologies). Chinese hamster ovary (CHO) cells transfected with the CD28 ligands CD80 or CD86 were the generous gift of G. Freeman (Dana-Farber Cancer Institute). CHO cells were grown in DMEM/F12 medium (Gibco, Fisher, Pittsburgh, PA) containing 10% heat-inactivated FCS; 100 U/ml penicillin; 100 μg/ml streptomycin; 10 mM HEPES, pH 7.2; 2 mM glutamine; 15 μg/ml gentamicin (Life Technologies); and 400 μg/ml geneticin (G418) (Life Technologies).

Antibodies

The CD3 mAb OKT3 (ATCC), CD5 mAb Leu 1 (Becton Dickinson, Mountain View, CA), CD7 mAb 3A1 (ATCC), CD28 mAb 9.6 (Mediatech, Herndon, VA) supplemented with 10% heat-inactivated FCS (Sigma, St. Louis, MO); 100 U/ml penicillin (Life Technologies, Grand Island, NY); 100 μg/ml streptomyycin (Life Technologies); 10 mM HEPES, pH 7.2 (BioWhittaker, Bethesda, MD); 2 mM glutamine (Life Technologies); and 50 μg/ml 2-ME (Sigma). Chinese hamster ovary (CHO) cells transfected with the CD28 ligands CD80 or CD86 were the generous gift of G. Freeman (Dana-Farber Cancer Institute). CHO cells were grown in DMEM/F12 medium (Gibco, Fisher, Pittsburgh, PA) containing 10% heat-inactivated FCS; 100 U/ml penicillin; 100 μg/ml streptomyacin; 10 mM HEPES, pH 7.2; 2 mM glutamine; 15 μg/ml gentamicin (Life Technologies); and 400 μg/ml geneticin (G418) (Life Technologies).

Preparation of cell lysates and immunoprecipitates

Jurkat cells (2 × 10⁷) were resuspended in 0.5 ml buffer A (RPMI 1640 supplemented with 100 U/ml penicillin; 100 μg/ml streptomycin; 10 mM HEPES, pH 7.2; and 2 mM glutamine) and incubated with 1 μg/ml mAb for 15 min on ice and then for 5 min at 37 °C. In some cases, Jurkat cells were stimulated with 1 × 10⁵ CHO cells or 2 μM pervanadate or pretreated with 5 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) or 10 μg/ml PD98059 (New England Biolabs, Beverly, MA), a selective inhibitor of MEK1 activation and the MAP kinase cascade. The cells were washed twice with ice-cold buffer A containing 1 mM sodium orthovanadate (Sigma) and then resuspended in lysis buffer (1% Nonidet P-40; 150 mM NaCl, 25 mM HEPES, pH 7.5; 1 mM EDTA; 1 mM sodium orthovanadate; 100 μg/ml soybean trypsin inhibitor; 10 μg/ml leupeptin; and 10 μg/ml aprotinin). The cell lysates (corresponding to 2 × 10⁷ cell equivalents for each immunoprecipitate) were incubated for 2 h at 4°C with 25 μl agarose-conjugated anti-phosphotyrosine mAb (Santa Cruz Biotechnology) or with 25 μl protein A-Sepharose (Pharmacia Biotech, Piscataway, NJ) and Abs to HS1, CD3, or CD28, as indicated. The immunoprecipitates were washed three times with lysis buffer and solubilized in SDS sample buffer, and the proteins were separated by electrophoresis on 6 to 15% SDS-polyacrylamide gel electrophoresis and transferred to poly(vinylidene difluoride) (PVDF) membranes (Millipore, Bedford, MA). Detergent lysates corresponding to 5 × 10⁶ cells were included in immunoblotting experiments.

Western blotting analysis

The PVDF membranes were blocked with buffer containing 20 mM Tris-HCl, pH 7.6; 150 mM NaCl; 0.05% Tween-20 (Sigma); and 1% BSA (Fraction V; Sigma) and then incubated sequentially with the indicated primary Ab followed by horseradish peroxidase-coupled secondary Ab (Amersham). The Ab-labeled protein bands were detected by autoradiography after enhanced chemiluminescence (Amersham) development.

NFAT luciferase assays

Jurkat T cells (1 × 10⁶) were incubated with 5 μg of an NFAT luciferase reporter plasmid containing a minimal IL-2 promoter downstream of three NFAT binding sites (27) for 15 min at room temperature and then pulsed in a Life Technologies electrophorator at 250 V and 800 μF. The cells remained at room temperature for 10 min and then were incubated for 24 h at 37°C in 10% RPMI. The transfected cells were stimulated for 6 h with the indicated mAbs, and then the samples were prepared using the Enhanced Luciferase Assay Kit (Analytic Luminescent Laboratory, San Diego, CA) according to the manufacturer’s instructions. NFAT luciferase activity was assessed with a Monolight 1010 lumimeter (San Diego, CA) and is presented as the fold activation relative to the basal level detected in unstimulated Jurkat cells transfected with the reporter plasmid.

Results

Engagement of CD3 but not of the costimulatory receptors CD28 or CD2 induced the tyrosine phosphorylation of HS1

HS1, a hematopoietic cell-specific signaling protein, is phosphorylated on tyrosine residues in response to ligation of Ag receptors on B cells (16, 28), T cells (21), and mast cells (17). We examined whether engagement of the T cell costimulatory receptor CD28, CD2, or other T cell surface receptors could induce the tyrosine phosphorylation of HS1 in Jurkat T cells. Anti-phosphotyrosine immunoprecipitates were prepared from Nonidet P-40 detergent lysates of cells that had been stimulated for 5 min at 37°C with mAbs to different T lymphocyte transmembrane receptor molecules or that had been chemically activated with pervanadate. The immunoprecipitated phosphoproteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with an HS1 polyclonal antiserum raised against a human HS1 peptide (Val306 to Ser329). As expected, stimulation of Jurkat cells with anti-CD3 mAb led to the immunoprecipitation of a tyrosine-phosphorylated 75-kDa protein that was specifically detected by anti-HS1 antiserum (Fig. 1A, lane 4). This phosphoprotein comigrated with the 75-kDa cell lysate protein detected by anti-HS1 antisera (Fig. 1A, lane 1) and was not found in anti-phosphotyrosine immunoprecipitates prepared from unstimulated cells (Fig. 1A, lane 2). The CD3-dependent induction of HS1 phosphorylation was detectable within 45 s of stimulation, maximal at 7 min, and notably decreased by 30 min (data not shown). Thus, stimulation of Jurkat T cells with anti-CD3 mAb was sufficient to induce the tyrosine phosphorylation of HS1, consistent with published studies in murine systems (21, 22). Stimulation of the cells with 2 μM pervanadate, a potent inhibitor of tyrosine phosphatase activity, also induced robust phosphorylation of HS1 on tyrosine residues (Fig. 1A, lane 10). In contrast, neither stimulation of the cells with anti-CD28 nor with anti-CD2 mAbs (Fig. 1A, lane 3) led to the tyrosine phosphorylation of HS1 (Fig. 1A, lane 7). Tyrosine-phosphorylated HS1 was also not detected in Jurkat cells that had been stimulated with mAbs to CD5, CD7, or CD43 (Fig. 1A, lanes 3, 5, 6, and 8).

Because stimulation of CD28 with its physiologic ligands CD80 and CD86 can recruit different intracellular signals from anti-CD28 mAb (Ref. 29 and our manuscript in preparation), we examined the effect of stimulating Jurkat T cells with these ligands.
hs1 and T cell signaling

Inducible gene expression is a crucial effector function of specific T cell signaling cascades. Antigenic stimulation of T cells induces the transcriptional activation of NFAT family members that are required for the production of IL-2 (31). We investigated whether HS1 was a component of the signaling cascades leading to NFAT activation and IL-2 production. Jurkat T cells (1 × 10^7) were transiently transfected with an NFAT luciferase reporter construct containing a minimal IL-2 promoter downstream of three NFAT binding sites. Transfected cells were stimulated with the anti-CD3 mAb OKT3, the anti-CD2 mAbs T112 and T113, or the anti-CD28 mAb 9.3 for 6 h before harvest and quantification of luciferase activity. Stimulation with mitogenic pairs of CD2 mAbs was sufficient to increase the amount of tyrosine-phosphorylated HS1 over the background levels present in unstimulated cells (Fig. 3A). CD28 costimulation enhanced both the CD3- and the CD2-dependent induction of NFAT luciferase activity. However, the response to combined stimulation through CD3 and CD2 was no greater than that seen to CD2 alone. In marked contrast to the NFAT assays, stimulation with either of two different mitogenic pairs of CD2 mAbs, T112 plus T113, or 9-1 plus 9.6, did not increase the amount of tyrosine-phosphorylated HS1 over the background levels present in unstimulated cells (Fig. 3B, lanes 2, 5, and 6). Combined stimulation with anti-CD2 and anti-CD28 mAbs (Fig. 3B, lanes 2, 8, and 10) failed to induce the tyrosine-phosphorylated HS1, despite their ability to induce robust NFAT transcriptional activation (Fig. 3A). The absence of HS1 phosphorylation in response to stimulation with mitogenic pairs of anti-CD2

No enhancement of HS1 tyrosine phosphorylation was observed in Jurkat cells that had been stimulated with CD80- or CD86-transfected CHO cells (Fig. 1, lanes 4–6) or in mock-CHO-stimulated (Fig. 1, lane 7) Jurkat cells. These stimulation conditions were sufficient to induce the physical association of p85 PI 3-kinase with CD28 (Ref. 10 and data not shown). Anti-HS1 antisera did not detect any 75-kDa proteins in anti-phosphotyrosine immunoprecipitates prepared from detergent lysates of the different CHO cell lines (Fig. 1B, lanes 1–3), consistent with the lack of expression of HS1 in these cells. Stimulation of the Jurkat cells with anti-CD28 mAb that was further cross-linked with secondary Ab (Fig. 1B, lane 9) failed to induce the tyrosine phosphorylation of HS1. Taken together, these results indicated that the signaling pathways triggered by engagement of the CD3 and either the CD28 or CD2 receptors on Jurkat cells can be distinguished by their differing capacity to induce the tyrosine phosphorylation of HS1.

We confirmed that our stimulation conditions with anti-CD28 and anti-CD2 mAbs were sufficient to induce activation-dependent changes in tyrosine phosphorylation of other intracellular signaling proteins. Ligation with anti-CD28 mAb led to the association of CD28 with the 85-kDa regulatory subunit of PI 3-kinase (Ref. 30 and data not shown) and the tyrosine phosphorylation of p95 Vav (see below) (30). Stimulation of the CD2 receptor induced the tyrosine phosphorylation of multiple other intracellular proteins (Fig. 2, left), including the specific phosphorylation of p85 PI 3-kinase (Fig. 2, right). These experiments illustrate the specificity of the responses triggered through the different cell surface receptors.

Activation-dependent tyrosine phosphorylation of HS1 is not required for induction of NFAT activity

The migration patterns of several inducibly tyrosine-phosphorylated proteins (arrows) and molecular mass markers are indicated.

FIGURE 1. CD3-dependent tyrosine phosphorylation of HS1. Anti-phosphotyrosine immunoprecipitates were prepared from Jurkat T cells (2 × 10^7). A, Jurkat T cells that were untreated (lanes 1 and 2) or stimulated with 1 μg/ml anti-CD2 mAbs T112 + T113 (lanes 3 and 9), the anti-CD3 mAb OKT3 (lane 4), the anti-CD5 mAb Leu 1 (lane 5), the anti-CD7 mAb 3A1 (lane 6), the anti-CD28 mAb 9.3 (lanes 7 and 9), the anti-CD43 mAb L10 (lane 8), or 2 μM pervanadate (lane 10). B, Jurkat T cells that were untreated (lane 7) or stimulated with mock-transfected CHO cells (1 × 10^7) (lane 4), CHO-CD80 cells (lane 5), CHO-CD86 cells (lane 6), 1 μg/ml anti-CD28 mAb 9.3 (lane 8), 1 μg/ml anti-CD28 mAb 9.3, and 5 μg/ml goat anti-mouse secondary cross-linking Ab (lane 9) or 1 μg/ml anti-CD3 mAb OKT3 (lane 10). Anti-phosphotyrosine immunoprecipitates were also prepared from the mock-transfected CHO cells (lane 1), CHO-CD80 cells (lane 2), and CHO-CD86 cells (lane 3) alone. All stimulations were for 5 min at 37°C. Proteins were separated on 6 to 15% SDS-PAGE gradient gels, transferred to PVDF membranes, probed with anti-HS1 antiserum, and detected by autoradiography after enhanced chemiluminescence treatment as described in Materials and Methods. The migration patterns of HS1 and molecular mass markers are indicated.

FIGURE 2. Induction of tyrosine phosphorylation by mitogenic pairs of anti-CD2 mAbs. Left, Jurkat T cells were untreated (lane 1) or were stimulated with 1 μg/ml of the anti-CD2 mAbs T112 and T113 (lane 2) or 1 μg/ml anti-CD3 mAb OKT3 (lane 3) for 5 min at 37°C. Tyrosine-phosphorylated proteins were detected by immunoprecipitation and blotting with anti-phosphotyrosine mAb. The migration patterns of several inducibly tyrosine-phosphorylated proteins (arrows) and molecular mass markers are indicated. Right, Jurkat T cells were untreated (lanes 1 and 2) or were stimulated with 1 μg/ml of the anti-CD2 mAbs T112 and T113 (lane 3) for 5 min at 37°C. Proteins in 1% Nonidet P-40 cell lysates (lane 1) or anti-phosphotyrosine immunoprecipitates (lanes 2–3) were immunoblotted with anti-p85 PI 3 kinase antiserum. The migration patterns of p85 PI 3 kinase and molecular mass markers are indicated.
phosphorylation (Fig. 4, lane 3) with anti-CD3 mAb OKT3 (lanes 3, 6, and 7), 1 μg/ml anti-CD3 mAb OKT3 (lanes 8–10), anti-CD2 mAbs T11 and T11, or anti-CD28 mAb 9.3 (lanes 5, 7, and 10) for 5 min at 37°C. Proteins in 1% Nonidet P-40 cell lysates (lane 1) or anti-phosphotyrosine immunoprecipitates (lanes 2–10) were immunoblotted with anti-HS1 antisera. The migration patterns of HS1 and molecular mass markers are indicated.

Stimulated, vector-transfected cells (assigned an arbitrary value of 1). The results of a single experiment are shown that are representative of two independent experiments. B, Jurkat T cells were left untreated (lanes 1 and 2) or were stimulated with 1 μg/ml anti-CD3 mAb OKT3 (lanes 3, 7, and 9), anti-CD28 mAb 9.3 (lanes 4, 8, and 10), anti-CD2 mAbs T11, and T11, (lanes 5, 7, and 8), or anti-CD2 mAbs 9.1 and 9-6 (lanes 6, 9, and 10) for 5 min at 37°C. Proteins in 1% Nonidet P-40 cell lysates (lane 1) or anti-phosphotyrosine immunoprecipitates (lanes 2–10) were immunoblotted with anti-HS1 antisera. The migration patterns of HS1 and molecular mass markers are indicated.

mAbs was particularly surprising, because CD2-dependent signaling pathways are often considered to be qualitatively similar to CD3-dependent pathways and, as we and others have shown, are sufficient to induce IL-2 production (32–35). These results clearly distinguish the conditions required for HS1 tyrosine phosphorylation from those required for activation of NFAT, suggesting that HS1 is not a necessary component of the signaling pathway leading to IL-2 production.

Neither of the costimulatory receptors CD28 or CD2 modulate the CD3-dependent phosphorylation of HS1

The costimulatory signals provided by CD28 and CD2 have not yet been fully characterized. We examined whether engagement of these receptors could potentiate or inhibit a suboptimal signal through CD3. Incubation of Jurkat cells with 10 ng/ml anti-CD3 mAb did not lead to any detectable increases in HS1 tyrosine phosphorylation (Fig. 4, lane 3). This basal level of HS1 phosphorylation was not augmented by costimulation through CD28 or CD2 (Fig. 4, lanes 6 and 7). Furthermore, costimulation with either anti-CD2 or anti-CD28 mAbs did not change the level of tyrosine-phosphorylated HS1 from that seen after stimulation with optimal concentrations of anti-CD3 mAb alone (Fig. 4, lanes 8–10).

Thus, in Jurkat T cells, the induction of HS1 tyrosine phosphorylation is exquisitely dependent upon the nature of the activating signal, occurring in response to engagement of CD3 but not CD28, CD2, CD5, CD7, or CD43. Ag receptor specificity has also been seen following engagement of the Ag receptors on B cells (16, 17, 28) and FcεRI on mast cells (17). Importantly, the costimulatory signal triggered by engagement of CD28 and CD2 did not appear to influence the level of HS1 phosphorylation.

Lck and CD45 are required for CD3-dependent tyrosine phosphorylation of HS1 in vivo

Studies in mice rendered genetically deficient in the Src family tyrosine kinase Lyn have shown that this kinase is required for activation-dependent phosphorylation of HS1 on tyrosine in response to engagement of the B cell Ag receptor (22). In vitro studies have also shown that the SH3 domain of the Src family tyrosine kinase Lck can bind HS1; however, this binding is independent of activation (18, 36). Given the importance of Lck and the tyrosine phosphatase CD45 in CD3-dependent signal transduction (37, 38), we examined whether these proteins were required for CD3-dependent tyrosine phosphorylation of HS1 in vivo using the Jurkat subclones J45.01 (deficient in CD45 expression) and J.Cam1 (deficient in Lck activity). The CD3-dependent tyrosine phosphorylation of HS1 was detectable only in the Jurkat cells and detectable amounts of HS1 phosphorylation in vivo using the Jurkat subclones J45.01 (deficient in CD45 expression) and J.Cam1 (deficient in Lck activity). The CD3-dependent tyrosine phosphorylation of HS1 in the Jurkat cells and detectable amounts of HS1 tyrosine phosphorylation in the J45.01 and J.Cam1 cells (Fig, 5, lanes 7, 11, and 15). All three cell lines expressed comparable amounts of HS1 (Fig. 5, lanes 1–3) as well as similar levels of CD3 and CD28 surface expression as determined by indirect immunofluorescence staining (data not shown). Since CD45 is required for normal Lck activity and CD3-dependent signaling (39), our in vivo results are consistent with the hypothesis that Lck tyrosine kinase activity is required for CD3-dependent tyrosine phosphorylation of HS1.

Phorbol ester treatment inhibited CD3-dependent tyrosine phosphorylation of HS1

Jurkat T cells produce IL-2 in response to treatment with either anti-CD3 or anti-CD28 mAb when given in conjunction with low.
Inhibition of activation-dependent HS1 tyrosine phosphorylation by PMA. Jurkat T cells were incubated with PMA (lanes 1 and 7) or with 5 ng/ml PMA (lanes 2 and 7–10) for 48 h. After harvest, the cells were left untreated (lanes 1–3) or stimulated with anti-CD28 mAb 9.3 (lanes 4, 6, 8, and 10) or anti-CD3 mAb OKT3 (lanes 5, 6, 9, and 10) for 5 min at 37°C. Proteins in 1% Nonidet P-40 cell lysates (lanes 1 and 2) or anti-phosphotyrosine immunoprecipitates (lanes 3–10) were simultaneously immunoblotted with anti-HS1 antiserum and anti-Vav antisera.

**Discussion**

Ligation of the TCR/CD3 complex induces the rapid tyrosine phosphorylation of HS1 in Jurkat T cells. HS1 phosphorylation required the activation of the Src family kinase Lck: we did not find CD3-dependent HS1 phosphorylation in Jurkat cells rendered genetically deficient in Lck kinase activity or in CD45 phosphatase activity (Fig. 5). This is in agreement with a recent report that whether a brief pretreatment with PMA affected CD3-dependent stimulation of HS1 tyrosine phosphorylation. Jurkat T cells were left untreated or pretreated with 5 ng PMA for 30 min at 37°C before stimulation with anti-CD3 mAb. The short incubation with PMA was sufficient to inhibit most of the tyrosine phosphorylation of HS1 in response to stimulation with either anti-CD3 (Fig. 7, top). Short-term PMA treatment did not inhibit CD3-dependent tyrosine phosphorylation of p95 Vav and did not affect expression of CD3 or HS1 (data not shown). Thus, both short- and long-term treatments with PMA decrease the CD3-dependent tyrosine phosphorylation of HS1. Furthermore, PKC appears to regulate the tyrosine phosphorylation of HS1 but not that of p95 Vav.

Activation of MEK1 is not required for CD3-dependent tyrosine phosphorylation of HS1

The intracellular signals triggered by engagement of CD3 include the phosphorylation of the MAP kinases Erk1 and Erk2 by the MAP kinase kinase MEK1 (9). We used PD98059, a selective inhibitor of MEK1 activation, to determine whether this kinase cascade was required for the tyrosine phosphorylation of HS1. Stimulation with anti-CD3 mAb induced the tyrosine phosphorylation of HS1 and the Erk kinases (Fig. 7, lane 3, top and bottom, respectively). Pretreatment of the cells for 30 min at 37°C with 10 μM of the MEK1 inhibitor was sufficient to inhibit more than 70% of the Erk phosphorylation (Fig. 7, lane 5, bottom) without diminishing the HS1 phosphorylation (Fig. 7, lane 5, top). In contrast, pretreatment of the cells with PMA for 30 min at 37°C inhibited the CD3-dependent phosphorylation of HS1 (Fig. 7, lanes 6 and 7, top), and PMA treatment alone was able to stimulate the tyrosine phosphorylation of the Erk kinases (Fig. 7, lanes 6 and 7, bottom). Thus, the tyrosine phosphorylation of HS1 after CD3 ligation is not dependent upon the activation of MEK1 or its downstream effector molecules.
CD45-null thymocytes also show impaired CD3-dependent phosphorylation of HS1 (41). Importantly, activation of Lck is not sufficient for induction of HS1 tyrosine phosphorylation in vivo. We found that tyrosine phosphorylation of HS1 was not detected after engagement of CD28 or CD2, despite the ability of these receptors to activate Lck and other Src family tyrosine kinases (42–47). Engagement of CD3, but not CD28 or CD2, activates the Syk family tyrosine kinase ZAP70 (48). Our data are consistent with a model whereby CD3-dependent tyrosine phosphorylation of HS1 requires the dual activation of both ZAP70 and Lck, a synergy that is not recruited by either the CD28 or CD2 receptor. This model is supported by reports that Syk and the Src family kinase Lyn are both required for HS1 phosphorylation in B cells (23, 49). Alternatively, engagement of the CD28 and/or CD2 costimulatory receptors may differentially activate protein tyrosine phosphatases, thereby inhibiting the tyrosine phosphorylation of HS1. It has been shown that SHP2 can associate with the CD28 family member CTLA-4 (50) and CD45 can associate with CD2 (51, 52). However, simultaneous stimulation of CD3 and CD28 or CD2 did not diminish the tyrosine phosphorylation of HS1, suggesting that the activity of a tyrosine phosphatase, if one exists, is not dominant under these conditions.

We also found that activation of PKC by PMA treatment preferentially inhibited the CD3-dependent tyrosine phosphorylation of HS1 but not that of Vav. PMA has multiple effects on intracellular signaling pathways (53). However, it is unlikely that inhibition of Lck activity by PKC causes the inhibition of HS1 phosphorylation, since the CD3-induced phosphorylation of Vav is also dependent upon Lck (54). Taken together, these findings suggest that HS1 and Vav are differentially regulated by PKC downstream of Lck. It is possible that phorbol ester treatment induces the serine/threonine phosphorylation of HS1, preventing its interaction with Lck. The proline-rich region of HS1, required for binding to the SH3 domain of Lck, contains a potential consensus site for phosphorylation by proline-directed serine/threonine kinases (18) activated by phorbol esters. Alternatively, PKC may inhibit activation of a required intermediate such as a Syk family kinase or modify the intracellular localization of HS1 or its partner proteins.

Importantly, our studies indicate that activation-dependent tyrosine phosphorylation of HS1 is not required for NFAT transcriptional activation. NFAT proteins are transcriptional regulators of critical immune response genes, including IL-2. There was no detectable tyrosine phosphorylation of HS1 after cells were stimulated with mitogenic pairs of CD2 mAbs, conditions sufficient for NFAT luciferase activity (Fig. 3A) and IL-2 production (5, 8). Furthermore, CD3-dependent tyrosine phosphorylation of HS1 was not diminished by incubation of the cells with cyclosporine (data not shown), a calcineurin inhibitor that prevents the nuclear translocation and transcriptional activation of NFAT (55, 56). Thus, phosphorylation of HS1 appears to be independent of the calcium arm of CD3-dependent signaling, which involves the protein tyrosine kinases Fyn and Itk (57, 58).

We also found that inhibition of MEK1 activity had no effect on CD3-dependent HS1 phosphorylation (Fig. 7). Thus, neither MEK1 nor its downstream effectors are directly required for activation-dependent tyrosine phosphorylation of HS1. Moreover, since MEK1 activation of the Erk kinases is an important step in CD3-dependent IL-2 production, this finding is consistent with the hypothesis that regulation of cytokine production is not a primary function of HS1 in T cells. In this regard, it is intriguing that HS1 has been implicated in apoptotic signaling pathways in B cells, although a comparable effect in T cells remains to be established. In B cells, HS1 can bind to HAX-1, a 35-kDa protein that shares sequence similarities with Bcl-2 family members and colocalizes with Bcl-xL in the mitochondria (59). In T cells, the survival signal transmitted even though CD28 involves the up-regulation of Bcl-xL expression and function. Whether Bcl-xL could in turn modulate a CD3-dependent apoptotic pathway mediated by the interaction of HS1 and HAX-1 in T lymphocytes remains to be tested.

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