Recruitment of the Actin-binding Protein HIP-55 to the Immunological Synapse Regulates T Cell Receptor Signaling and Endocytosis*

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Actin cytoskeleton dynamics critically regulate T cell activation. We found that the cytoplasmic adaptor HIP-55, a Src/Syk-kinases substrate and member of the drebrin/Abp1 family of actin-binding proteins, localized to the T cell-antigen-presenting cell (APC) contact site in an antigen-dependent manner. Using green fluorescent protein fusion proteins, both Src homology 3 (SH3) and actin binding domains were found necessary for recruitment at the T cell-APC interface. HIP-55 was not implicated in conjugate formation and actin polymerization but regulated distal signaling events through binding and activation of hematopoietic progenitor kinase 1 (HPK1), a germline center kinase (GCK) family kinase involved in negative signaling in T cells. Using RNA interference and overexpression experiments, the HIP-55-HPK1 complex was found to negatively regulate nuclear factor of activated T cell (NFAT) activation by the T cell antigen receptor. Moreover, we show that HIP-55, which partly co-localized with early endocytic compartments, promoted both basal and ligand-dependent T cell receptor (TCR) down-modulation, resulting in a decreased TCR expression. SH3 and actin-depolymerizing factor homology domains were required for this function. As controls, the expression of CD28 and the glycosylphosphatidylinositol-linked protein CD59 was not affected by HIP-55 overexpression. These results suggest that, in addition to binding to HPK1, HIP-55 might negatively regulate TCR signaling through down-regulation of TCR expression. Our findings show that HIP-55 is a key novel component of the immunological synapse that modulates T cell activation by connecting actin cytoskeleton and TCRs to gene activation and endocytic processes.

A critical event in the initiation of the adaptive immune response is the activation of T lymphocytes. Initial TCR1 ligation by cognate antigen induces sequential activation of protein tyrosine kinases of the Src (Lck and Fyn) and Syk (ZAP-70 and Syk) families that initiates the assembly of multimolecular signaling complexes (1). These events lead to the formation of spatially organized supramolecular activation clusters (SMACs) polarized at the T cell-APC interface in a macrostructure termed the immunological synapse (IS) (2). A dynamic reorganization of the actin cytoskeleton in response to TCR and co-stimulatory receptor engagement is required for the formation of a mature IS (3, 4). Indeed, actin remodeling accompanies early activation events and is likely to be required for most of them to take place (5). Cytoskeletal dynamics also appear to be involved in the specific patterning of negative regulatory receptors that fine-tune T cell biological responses (6, 7). Evidence suggests that IS formation correlates with transcription of cytokine genes, cellular proliferation, and effector cell differentiation (8). However, the exact function of the IS is still unclear, and it has been proposed that the IS serves as a site where clustered TCRs could be subjected to endocytosis, allowing TCR signaling down-regulation (8–10).

Several actin-binding proteins have been involved in the process of T cell activation mediated by APCs. One of the best studied examples is the complex formed by the Wiskott-Aldrich syndrome protein (WASP) with the actin-related protein Arp2/3 (11). This complex is activated following the phosphorylation of the adaptor SLP-76 by activated ZAP-70. SLP-76 then associates with Nck (12) and Vav1 (13), a guanine exchange factor that promotes the activation of Rho GTTPases and their effectors, including WASP. The WASP-Arp2/3 complex then regulates actin nucleation, resulting in the extension and branching of the actin filaments. Genetic studies have shown that WASP- and Vav1-deficient T cells exhibit TCR clustering and activation defects following TCR engagement (14, 15), demonstrating their importance in the formation of a mature IS. However, the function of several other classes of actin cytoskeleton regulatory proteins expressed in lymphocytes is currently unclear (for review, see Ref. 16).

We have investigated the involvement during T cell activation of the cytoplasmic adaptor protein HIP-55 (also called SH3P7 or mAbp1), a member of the drebrin/Abp1 class of cellular signal-regulated kinase; FACS, fluorescence-activated cell sort-er; FITC, fluorescein isothiocyanate; GCK, germinal center kinase; GFP, green fluorescent protein; HA, hemagglutinin A; HPK1, hematopoietic progenitor kinase 1; IL, interleukin; IS, immunological synapse; mAb, monoclonal antibody; NFAT, nuclear factor of activated T cells; OVA, ovalbumin-derived (peptide); PMA, phorbol 12-myristate 13-acetate; SEC, staphylococcal enterotoxin E; SH, Src homology; siRNA, small interfering RNA; TRITC, tetramethylrhodamine isothiocyanate; WASP, Wiskott-Aldrich syndrome protein.
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actin-binding proteins. The drebrin/Abp1 family is conserved from yeast to mammals and is characterized by the presence of a homologous N-terminal actin-depolymerizing factor homology (ADF-H) domain (17, 18). This family includes SH3P7 in mouse (19, 20) and Abp1 in yeast (17). The ADF-H domain is also found in proteins that regulate the disassembly of actin filaments such as cofilins and twinfilins (18). The drebrin/Abp1 family, which binds F-actin but not actin monomers, is involved in signaling events in multiple organisms and cells. For example, the yeast Abp1 is connected to endocytic trafficking and Arp2/3 activation (21), whereas drebrins participate in cytoskeleton plasticity and morphogenesis in neurons (22).

HIP-55 is composed of an N-terminal ADF-H domain, two conserved tyrosine phosphorylation sites, and a C-terminal SH3 domain and has no activity of actin polymerization (19, 23). The homologue of HIP-55 in mouse was found to be a substrate of Src and Syk family kinases following B-cell receptor stimulation (20), suggesting that it may represent a common effector of antigen receptor-signaling pathways in leukocytes. Moreover, HIP-55 associates with dynamin, a GTPase that functions in endocytosis, indicating that it may also connect the actin cytoskeleton to endocytic function (24, 25). Finally, HIP-55 was cloned as a hematopoietic progenitor kinase 1 (HPK1)-interacting protein (23), an association that suggests HIP-55 involvement in the regulation of HPK1 activity and localization. HPK1 is a member of the GCK family (26), which is involved in TCR and B cell receptor signaling (27, 28). HPK1 function in T cells is unclear. However, overexpression of HPK1 inhibits ERKs and AP-1 activities following TCR cross-linking (27). Thus, the drebrin/Abp1 family represents a novel group of signaling molecules with connecting functions between cellular trafficking, signal transduction, and cytoskeletal organization.

In this study, we show that antigen-dependent TCR engagement results in the accumulation of HIP-55 at the T-cell-APC contact site. Using a combination of confocal microscopy, RNA interference, and overexpression experiments, we show that HIP-55 does not contribute to the early formation of T-cell-APC conjugates. However, we found that HIP-55 is a key component of the immunological synapse that regulates T cell activation by bridging TCRs and the actin cytoskeleton to gene activation and endocytic processes.

EXPERIMENTAL PROCEDURES

Mice and Reagents—Homozygous Do11.10 mice were a gift from Dr. S.D. Hurst (DNAX Research Institute, Palo Alto, CA). Recombinant mouse IL-12 was purchased from BD Pharmingen. Ovalbumin-derived (OVA) peptide (323–339), TRITC- and FITC-conjugated phalloidin were purchased from Molecular Probes (Eugene, OR). Other chemicals were from Sigma.

Plasmids—The NFAT and IL-2 promoter luciferase reporter plasmids were obtained as described previously (29). The vectors encoding hemagglutinin A (HA)-tagged wild-type and kinase-dead HPK1 were a gift of Dr. F. Kiefer (Max Planck Institute, Germany) (26). Full-length HIP-55 cDNA and mutants with deletion of the SH3 or ADF-H domain were obtained from Sigma. Steaplycococcal enterotoxin E (SEE) was from Toxin Technologies (Madison, WI). Anti-CD3 monoclonal antibody X3 was purified from hybridoma supernatant. Anti-WASP was from Santa Cruz Biotechnology (Santa Cruz, CA), phosphatidyethanolamine-conjugated goat anti-mouse was from Dako (Copenhagen, Denmark), Cy5-conjugated donkey anti-mouse and FITC-conjugated goat anti-rabbit were from Jackson ImmunoResearch Laboratories, and FITC-conjugated anti-mouse CD43, anti-mouse CD4, anti-mouse CD8α, and anti-mouse CD90 were from BD Pharmingen. Cell Tracker Orange (CMTOFR) was obtained from Molecular Probes (Eugene, OR). Other chemicals were from Sigma.

Cell Culture and Transfection—CD4<sup>+</sup> CD62L<sup>−</sup> naive T cells from DO11.10 mice were purified from spleen and sorted by positive selection. Cells were then incubated with irradiated spleen cells (0.5 μg/ml OVA peptide (323–339), 100 IU/ml IL-12, and 10 μg/ml anti-IL-4 mAb for 1 week as described previously (30). Mouse A20 B lymphoma cells were kindly provided by Dr. C. Fournier (INSERM U477, Paris, France). Human leukemia Jurkat T cells and simian virus 40 large T antigen-transfected Jurkat (Jurkat TAg) cells were cultured and transfected as described (29). Raji and HD-1 clones were from the American Type Culture Collection and were cultured as Jurkat cells. To establish stably transfected Jurkat cells, 2 days after electroporation with pEGFP-N1, pEGFP-N1-HIP-55, pEGFP-N1-ADF-H and pEGFP-N1-ΔSH3, 1 mg/ml G418 was added to the culture media. After 1 month, drug-resistant clones were isolated by limiting dilution, and the protein levels of the clones were determined by Western blotting.

Cell Culture and Transfection—CD4<sup>+</sup> CD62L<sup>−</sup> naive T cells from
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Stably transfected Jurkat cells were prepared in ice-cold lysis buffer and Western blotted as described in the previous section. Flow Cytometry Analysis of TCR-CD3 Down-regulation—Stably transfected Jurkat cells were either left unstimulated or stimulated with anti-CD3 (2 μg/ml) mAb for 1 h at 37 °C. Cells were then transferred to ice-cold PBS containing 1% BSA and 0,1% NaN₃ and washed twice. Collected cells were stained with a phosphatidylethanolamine-conjugated goat anti-mouse and analyzed on a FACScan flow cytometer. As shown in the formula labeled Equation 1.

\[
\text{%CD3 expressed} = \frac{\text{MFI at time} \div \text{MFI of vector expressed cells at time 0}) \times 100}{(\text{Eq. 1})}
\]

CD3 expression was calculated with gated GFP⁺ T cells.

RESULTS

HIP-55 Is Recruited to the T cell-APC Contact Site in Activated T Cells—to investigate HIP-55 involvement in T cell activation, we first studied, using confocal microscopy, its distribution during the formation of conjugates between DO11.10 OVA-specific TCR-transgenic CD4⁺ T cells and A20 B cells pulsed with the OVA peptide. In the absence of antigen, HIP-55 was homogeneously distributed throughout subcortical regions of the T cell cytoplasm (Fig. 1A). Following antigen stimulation, a time-dependent relocalization of HIP-55 was observed, and, at 10 min, a clear enrichment of HIP-55 molecules at the center of the contact area was observed (indicated by the white arrowheads in Fig. 1A). Between 10 and 30 min, HIP-55 was still enriched along the T cell-APC contact region referred to as mature synapse. After 60 min of activation, the cluster of HIP-55 molecules had retracted to a smaller area in the center of the IS.

We next compared the localization of HIP-55, CD43, and WASP. CD43 is a transmembrane glycoprotein found at the periphery of the T cell-APC junction (33), whereas WASP accumulates at the T cell-APC interface (31). Thus, these two proteins could be used as markers of different regions of the IS. In the absence of antigen, CD43 was uniformly distributed on the T cell surface, whereas WASP was occasionally seen enriched in the T cell-APC contact site and at the opposite pole of the contact zone. In the presence of antigen, both HIP-55 and WASP re-localized to the center of the contact zone. This clearly contrasted with CD43, which was segregated at the periphery of the IS. When randomly selected conjugates were scored for the presence or absence of HIP-55 at the site of contact, we found that ~70% of the conjugates formed in the presence of antigen showed HIP-55 accumulation, whereas, in the absence of antigen only, 2–3% of conjugates showed accumulation (Fig. 1C). These results show that HIP-55 is relocalized to the T cell-APC junction in an antigen-dependent manner.

HIP-55 Targets the T Cell-APC Contact Site via Its SH3 and ADF-H Domains—We sought to investigate which domains of HIP-55 are important for IS recruitment by using Jurkat cells. U937 monocytic cells armed with Fc receptor-bound anti-CD3 antibodies formed conjugates with Jurkat cells in which endogenous HIP-55 was found to accumulate at the contact site (Fig. 2A), therefore making this experimental system amenable for carrying out structure-function analysis of the HIP-55 molecule. Two GFP-tagged HIP-55 mutants were generated: (i) the ΔADF-H mutant lacking the first 146 amino acids containing the ADF-H domain that binds filamentous actin (F-actin) (19); and (ii) the ΔSH3 mutant lacking the C-terminal SH3 domain that interacts with the proline-rich sequences (Fig. 2B). The subcellular distribution of GFP fusion proteins transiently expressed in Jurkat T cells engaged in anti-CD3-dependent conjugates was then analyzed. In unstimulated cells, GFP-HIP-55 and GFP-ΔADF-H mutant proteins were homogeneously distributed at the plasma membrane, in contrast to the cytoplasmic and nuclear distribution of GFP-ΔSH3 mutant proteins (Fig. 2C). Upon stimulation, whereas the majority of the GFP-HIP-55 proteins redistributed to the central T cell-APC contact zone, the relocalization of both ΔADF-H and ΔSH3 mutant proteins was affected, because a significant proportion of mutant proteins accumulated at the periphery of the T cell-APC contact zone (Fig. 2C). These data indicate that both the SH3 and F-actin binding domains of HIP-55 are required for its targeting to the T cell-APC interface.

HIP-55 Negatively Regulates TCR-induced IL-2 Promoter Activation but Not the Formation of T Cell-APC Conjugates and Actin Polymerization—We asked whether wild-type or mutant HIP-55 proteins could affect the formation of stable T cell-APC conjugates. We adapted a FACS-based assay (32) to measure the ability of Jurkat cells transfected with GFP fusion proteins to form conjugates with U937 cells. Conjugates were formed as described in Fig. 2A and then analyzed by dual flow cytometry. The quantification of these experiments showed that the percentage of control-transfected T cells in conjugates increased 3–7-fold in the presence of anti-CD3 mAb (Fig. 3A). Jurkat cells transfected with ΔADF-H and ΔSH3 mutant proteins formed conjugates with the same efficiency as wild-type HIP-55-transfected Jurkat cells in the presence of anti-CD3 mAb. The only noticeable effect of ΔADF-H and ΔSH3 protein overexpression was a slight increase of the basal number of conjugates observed in the absence of anti-CD3 mAb (Fig. 3A). Because actin polymerization is an important event during conjugate formation, we therefore examined whether HIP-55 could interfere with actin polymerization induced by TCR engagement. Jurkat cells were transfected as described above and stimulated with anti-CD3 mAb for different periods of time. F-actin was then measured by phalloidin staining of fixed cells. Overexpression of wild-type and mutant HIP-55 proteins had no major effects on either basal or stimulated F-actin content (Fig. 3B).

Engagement of the TCR leads to transcriptional activation of cytokine genes. We thus examined whether HIP-55 could regulate distal signaling events such as TCR-induced IL-2 promoter activity. Jurkat cells were transfected with wild-type HIP-55 or the two mutant proteins and a luciferase reporter driven by the complete IL-2 promoter (Fig. 3C). Cells were stimulated with a combination of anti-CD3 mAb and sub-optimal concentration of PMA, and luciferase activities were determined. Expression of wild-type HIP-55 resulted in a dramatic reduction of the IL-2 promoter activity induced by TCR stimulation. In contrast, overexpression of ΔSH3 HIP-55 had no effect on the TCR-induced IL-2 promoter activity, whereas overexpression of ΔADF-H HIP-55 significantly increased it. These data indicate that HIP-55 negatively regulates IL-2 promoter activity in T cells and that both SH3- and ADF-H-dependent interactions are required for HIP-55 to function properly.

Physical and Functional Interaction between HIP-55 and HPK1—HIP-55 was cloned as an HPK1-interacting protein in fibroblasts (23), suggesting that HPK1 might associate HIP-55 at the T cell-APC interface. The binding of HIP-55 to HPK1 was studied in Jurkat cells either left unstimulated or stimulated by anti-CD3 mAb for 5 min. Cell lysates were precipitated using the anti-HIP-55 antibody, and the anti-HPK1 immunoblot revealed that HIP-55 interacted with HPK1 in both resting and stimulated cells (Fig. 4A). The localization of HIP-55 and HPK1 was next examined in conjugates formed between Jurkat cells and SEE-pulsed Raji B cells. In the absence of SEE, HIP-55 and HPK1 were evenly distributed throughout subcortical regions of T cell and APC cytoplasm, and FITC-conjugated phalloidin homogeneously stained a sub-cortical mem-

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brane ring (Fig. 4B). In the presence of SEE, HIP-55 and F-actin in T cells accumulated at the T cell-APC interface (Fig. 4B, arrowheads). A similar redistribution of HPK1 to the T cell side of the cell-cell contact zone was also observed. These results indicate that HIP-55 and HPK1 were recruited to the T cell-APC contact site in activated T cells and may participate in the same signaling pathway. We therefore compared their effect on NFAT transcriptional activity that promotes expression of the IL-2 gene. NFAT luciferase reporters were transfected with wild-type, kinase-inactive, HA-tagged HPK1 or V5-tagged HIP-55 alone or together. Cells were then stimulated with SEE-pulsed Raji B cells, and luciferase activities were measured (Fig. 4C). Individual overexpression of HIP-55 and HPK1 led to the inhibition of NFAT transcriptional activities following TCR engagement by SEE (2.4- and 3.2-fold inhibition, respectively). This effect was further increased by expression of
both proteins, resulting in a 6.5-fold inhibition of SEE-induced NFAT activation compared with cells transfected with empty vectors. In contrast, the expression of a kinase-inactive HPK1 (HPK1-K46E) significantly increased both basal and TCR-stimulated NFAT activities. Although the effect of HPK1-K46E on NFAT activities was significantly reduced by HIP-55 co-expression (Fig. 4C), HIP-55 overexpression did not result in a significant modification of the localization of HPK1-K46E compared with the wild-type protein (Fig. 4D). Our data strongly support the notion that HIP-55 and HPK1 physically interact to negatively regulate pathways leading to NFAT transcriptional activities in T cells.

**RNA Interference of HIP-55 and HPK1 Increases TCR Signaling to NFAT**—To further confirm the role of the HIP-55-HPK1 complex in NFAT activation, we used RNA interference (RNAi). We used this approach previously to knock down HIP-55 expression in human embryonic kidney cells (25). To obtain cells with homogeneous protein inhibition, Jurkat cells were co-transfected with duplexes of either siRNA HIP-55 or siRNA HPK1 in the presence of a CD20 expression plasmid and then purified by a magnetic bead-based procedure. Immunoblot analysis shows that CD20-positive Jurkat cells that had received siRNA HIP-55 or siRNA HPK1 reduced specifically the expression of the HIP-55 and HPK1 proteins, respectively, without affecting the expression of other proteins (Fig. 4E). As an additional control, scramble siRNAs had no effect on the expression of either protein. Quantification of the immunoblot signals showed that HIP-55 expression was reduced up to 70%, whereas HPK1 expression was reduced up to 95% (data not shown). In treated cells, NFAT luciferase activity revealed that the reduction of HIP-55 and/or HPK1 expression resulted in a strong increase of NFAT transcriptional activities induced by TCR cross-linking (1.5-, 2.3-, and 2.8-fold increase, respectively), compared with scramble siRNA-treated cells (Fig. 4F). Together with overexpression experiments, these data suggest that HIP-55 and HPK1 have a negative role in TCR-mediated activation and that a complex formed by these two proteins is required for such an effect.

**HIP-55 Co-localizes with TCR/CD3 and EEA1-labeled Endosomes in Resting and Stimulated Cells**—As shown in Fig. 4C,
Fig. 3. HIP-55 negatively regulates TCR-induced IL-2 promoter activation but not the formation of conjugates and actin polymerization. A, HIP-55 is not required for the formation of T cell-APC conjugates. Jurkat cells were transfected with GFP-tagged HIP-55 constructs. Cell conjugates were formed as described in Fig. 2 and then analyzed by dual flow cytometry. Conjugates appear in the upper right quadrant. Nb, number.

B, HIP-55 is not necessary for actin polymerization. Jurkat cells transfected with GFP-tagged HIP-55 constructs were incubated with anti-CD3 mAb (5 μg/ml) for the indicated times at 37 °C, fixed, labeled with TRITC-phalloidin, and analyzed by flow cytometry. Values represent the mean of fluorescence intensity (MFI) of triplicate determinations.

C, the ADF-H and SH3 domains are required for HIP-55 function. Jurkat cells were transfected with empty vector or with wild-type HIP-55 or ΔADF-H and ΔSH3 mutants in the presence of the IL-2 promoter reporter. Luciferase activity was determined in unstimulated cells (□) or in cells stimulated with anti-CD3 mAb (5 μg/ml) plus 10 ng/ml PMA (■). Expression of proteins was analyzed by anti-V5 or anti-actin immunoblots.
Fig. 4. Physical and functional interaction between HIP-55 and HPK1. A, Jurkat cells were left unstimulated (−) or stimulated (+) with anti-CD3 mAb for 5 min. Lysates were immunoprecipitated with the anti-HIP-55 antibody, and physical interaction was analyzed by anti-HPK1 and anti-HIP-55 immunoblots. B, Raji cells labeled with CMTMR were pulsed with SEE (5 μg/ml) for 30 min or left untreated (−SEE). T and Raji cells were then mixed and stained with FITC-phalloidin to visualize F-actin or with antibodies to HIP-55 or HPK1. Arrowheads indicate HIP-55, HPK1, and F-actin accumulation at the contact site. C, HIP-55 and HPK1 cooperate to inhibit NFAT activity. Jurkat cells were transfected with
against markers (all in antibody (green), anti-CD3 mAb (blue), and monoclonal antibody B). Fig. 3. Confocal microscopy was then performed using an anti-HIP-55 antibody (24, 25). We therefore examined the involvement of HIP-55 in T

functions in endocytic processes affecting receptor trafficking. First, we sought to compare, by confocal microscopy, the cellular localization of HIP-55 and the TCR-CD3 complex with known markers of sub-cellular compartments, including the early endosome antigen 1 (EEA1) and the cis-Golgi marker GM130. T cells were stimulated or not with SEE-pulsed, Raji B cells, fixed, and labeled with antibodies against HIP-55, the CD3 complex, and EEA1 or GM130. In unstimulated T cells, a significant proportion of cytoplasmic HIP-55 co-localized with EEA1—an early endosome and with the CD3 complex, whereas no HIP-55 was seen in GM130—cis-Golgi (Fig. 5). As shown in Figs. 1 and 4, after supernatant stimulation, HIP-55 relocated to the T cell-APC contact site. However, an important fraction of HIP-55 staining co-distributed with the CD3 complex and the EEA1-labeled endosomes found at the cell-cell interface. These results strongly point to a function of HIP-55 in the regulation of TCR endocytosis in both resting and stimulated T cells.

HIP-55 Overexpression Specifically Down-regulates TCR/CD3 Expression—Recent studies have shown that TCR complexes are continually recycling between the cell surface and the endocytic compartments (34), suggesting that the fraction of HIP-55 found co-localized with endosomes may participate in this process. To further examine the role of HIP-55 during TCR endocytosis, we established Jurkat cell clones stably transfected with HIP-55, ΔADF-H, or ΔSH3 mutants. Surface expression of CD3, CD28, and the glycosylphosphatidylinositol-linked protein CD59 was then analyzed in different clones (Fig. 6). A mix of Jurkat cell clones stably transfected with empty vector was used as the control. Expression of wild-type HIP-55 resulted in a dramatic reduction of CD3 expression (62 and 45% decrease compared with control for clone 8 and clone 9, respectively), whereas expression of both HIP-55 mutants increased it. By contrast, neither wild-type nor mutant HIP-55 proteins had any significant effect on CD59 expression, and only a slight increase of CD28 surface expression was observed on clones overexpressing HIP-55. Control immunoblots showed that each transfected protein was equally expressed. These data suggest that HIP-55 overexpression may specifically down-modulate TCR-CD3 expression by interfering with the constitutive recycling of TCR complexes. We therefore examined the effect of HIP-55 expression on ligand-dependent TCR down-modulation on a mixed population of representative clones. Cells were either left unstimulated or stimulated with anti-CD3 antibody for 1 h, and changes in CD3 cell surface expression were determined by flow cytometry. Stimulation led to a small but significant increase of CD3 down-modulation on HIP-55 transfected cells compared with control (Fig. 7A). In contrast, ΔSH3 mutant proteins had no effect on ligand-induced TCR down-modulation, whereas ΔADF-H mutants slightly attenuated it. Expression analysis showed that endogenous HIP-55 and transfected proteins were equally expressed (Fig. 7B). A quantification of the CD3 expression obtained from three independent experiments was represented in Fig. 7C. These data indicate that HIP-55 may negatively regulate T cell activation by promoting both ligand-independent and -dependent down-modulation of TCR/CD3 expression.

HIP-55 was still able to decrease NFAT activation in the presence of a kinase-inactive HPK1, suggesting that HIP-55 may act through several mechanisms to regulate TCR signaling. We and others have shown that, in non hematopoietic cells, HIP-55 functions in endocytic processes affecting receptor trafficking (24, 25). We therefore examined the involvement of HIP-55 in T cell endocytic pathways regulating TCR trafficking. First, we

empty vector, HPK1-HA, HIP-55-V5, and HPK1-HA-K46E alone or together in the presence of NFAT reporter construct. Luciferase activity was determined in cells stimulated for 6 h with Raji cells pulsed [●] or not [□] with 0.01 μg/ml SEE. Expression of proteins was analyzed by anti-V5 or anti-HA immunoblots. D, Jurkat cells were transfected by HIP-55 in the presence of either wild-type HPK1 or HPK1-K46E. Cells were then incubated with Raji cells labeled with CMTMR pulsed or not with SEE as described for Fig. 4B. Conjugates were then fixed and incubated with anti-HA antibodies to label HPK1 proteins. Arrowsheads indicate HPK1 and HPK1-K46E accumulation at the contact site. E, Jurkat cells were transfected with the indicated siRNAs and CD20 expression vector as described under “Experimental Procedures,” and protein expression was analyzed by immunoblotting with antibodies against HIP-55, HPK1, and ERK2. F, RNA interference of HIP-55 and HPK1 expression increases TCR signaling to NFAT. Jurkat cells were transfected as above in the presence of an NFAT reporter construct, and luciferase activity was determined in unstimulated cells [□] or in cells stimulated with anti-CD3 mAb [●]. Values are represented as the percentage of maximal values obtained by PMA plus ionomycin stimulation.
DISCUSSION

The actin cytoskeleton plays an essential role during the formation of the IS and T cell activation by stimulatory APCs (5, 16). The purpose of this work was to investigate the function of HIP-55, a member of a newly identified class of actin-binding proteins, the drebrin/Abp1 family, during T cell signaling. We show here that HIP-55 (mAbp1/SH3P7) constitutes a critical link between the TCR and both gene activation and endocytosis, two processes that critically affect T cell responsiveness. The appearance of a highly organized T cell-APC synapse correlates with the potent manifestation of T cell early activation events. A role of HIP-55 during the early steps of T cell stimulation by APCs is unlikely, because we found that overexpression of HIP-55 in T cells did not interfere with actin polymerization induced by TCR stimulation or with the early formation of T cell-APC conjugates. These findings are consistent with our data indicating that morphology and Rac1-induced actin polymerization in 293T cells were unaltered in the absence of HIP-55 (25). One explanation is that, in contrast to yeast Abp1, HIP-55 lacks a domain involved in stimulating actin nucleation activity of the Arp2/3 complex (21). However, our data identified HIP-55 as a novel component of the IS formed between T cells and APCs. The cellular model system utilized in our study reproduced the main features of a mature IS in that in its central area receptor-ligand pairs and intracellular signaling molecules, such as Lck, PKC-6, and WASP, were accumulated, whereas the peripheral zone was enriched in molecules like LFA-1 and CD43 (35). Comparison with CD43 and WASP distribution in resting and activated T cells shows that HIP-55 appeared more concentrated in the center of the IS. A relocalization of HIP-55 was induced by antigen-dependent and superantigen-induced TCR engagement. Similar results were found when we used GFP-transfected T cells conjugated with U937 monocytes cells loaded with anti-CD3 antibodies to mimic T cell-APC interaction. Moreover, tyrosine phosphorylation of mouse HIP-55 following T cell receptor and B cell receptor engagement has been reported (20), and we found that, following TCR stimulation, HIP-55 binds and is a substrate of Fyn (data not shown). These results further support the notion that HIP-55 is an effector of TCR-mediated signaling pathways, which localize to the T cell-APC interface upon antigenic stimulation.

How HIP-55 is targeted to the T cell-APC contact zone? The formation of T cell-APC conjugates involves a Rac1/Cdc42-WASP pathway leading to Arp2/3 activation and actin polymerization at the T cell-APC contact site (14, 36). In mouse fibroblasts, stimulation of Rac1 leads HIP-55 to accumulate to F-actin-rich lamellipodia at sites enriched for the Arp2/3 complex (19). Consistently, we observed that the recruitment of HIP-55 to areas of the IS enriched in polymerized actin requires a functional F-actin domain. Interactions with other signaling proteins through its SH3 domain are apparently also involved in HIP-55 relocalization. We show here that HIP-55 recruitment was concomitant with that of HPK1, a member of the GCK family, linked to TCR-dependent negative signaling pathways and interacting with Gads and SLP-76 adapters (27, 28). It is tempting to speculate that the recruitment of HIP-55 to the contact zone may involve its interaction with HPK1 bound to other adaptors such as SLP-76 or Gads. However, alternative ways for HIP-55 to translocate to the IS cannot be excluded. Indeed, we found a SH3-dependent binding of HIP-55 to two other important elements of TCR signaling, Fyn and Cbl (data not shown). Further studies are needed to precisely elucidate how HIP-55 is recruited to activated TCR complexes. However, our results indicate that mul-

FIG. 6. Expression patterns of surface molecules on T cells expressing HIP-55, ΔADF-H, or ΔSH3 mutants. Surface expression of CD3, CD28, and CD59 is shown. Jurkat cell stably expressed empty vector, HIP-55, ΔADF-H, or ΔSH3 were generated, and different clones (cl.) were analyzed as follows: 8 and 9 for HIP-55; 3 and 5 for ΔADF-H; 5 for ΔSH3; and vector for mixed population. Cells were washed in PBS-BSA-Na3, resuspended at 10^6 cells/ml, and labeled with CD3, CD28, and CD59 antibodies. After washes, cells were stained with a phycoerythrin secondary antibody and analyzed by flow cytometry. Expression of proteins was analyzed by anti-V5 immunoblot.
Multiple interactions with polymerized actin and SH3-binding signaling molecules are involved.

What is the function of HIP-55 during T cell activation? TCR signaling involves the coordinated function of multiple biochemical pathways that regulate complex cellular processes, such as membrane and cytoskeleton reorganization and intracellular trafficking of activated receptors and signaling molecules. Notably, the exact molecular composition of the pathways regulating down-modulation of the signals have remained elusive until recently (37, 38). We have shown here that RNAi-mediated silencing of HIP-55 expression in T cells significantly enhanced gene activation induced by TCR engagement, whereas HIP-55 overexpression decreased it. Thus, HIP-55 represents a novel negative regulator of antigen-dependent T cell activation. Interestingly, we and others have observed that overexpression of SH3P7, the mouse homologue of HIP-55, also reduced NFAT activation by TCR (our data not shown). HIP-55 might control T cell signaling by helping to localize and/or regulate the activity of HPK1 at the T cell-APC interface. HPK1 negatively regulates ERK and AP-1 activation following TCR stimulation (27, 28). Importantly, we observed that the binding of HIP-55 to HPK1 increased TCR-stimulated HPK1 kinase activity (data not shown), consistent with studies in non-hematopoietic cells (23). Both proteins were found localized in the T cell-APC contact zone, and we have shown that overexpression of either HPK1 or HIP-55 resulted in a dramatic reduction of gene activation induced by TCR engagement. On the contrary, reduction of HIP-55 or HPK1 expression by RNA interference or overexpression of kinase-deficient HPK1 increased TCR-mediated NFAT activation induced by TCR engagement. Gene silencing of both proteins resulted in an even more drastic phenotype, suggesting that the two proteins physically and functionally cooperate in a common pathway.

Alternatively, HIP-55 might regulate T cell activation by modulating TCR trafficking and expression. Recent studies have shown that TCR complexes are continually recycling between the cell surface and endocytic compartments (34). Upon antigenic stimulation, activated TCRs and associated signaling proteins are thought to be targeted for retention/degradation, leading to the down-modulation of TCR expression and signals (34, 39). The actin cytoskeleton plays a pivotal role during T cell activation, but its involvement in TCR endocytosis remains unclear. We and others have shown that HIP-55 regulates endocytic processes in non-immune cells (24, 25). In yeast, where the endocytic process and the actin cytoskeleton have been linked by genetic studies, Abp1 clearly functions in receptor endocytosis (17, 40). We have observed here an important reduction of CD3 expression on T cells overexpressing wild-type HIP-55 but not ADF-H and SH3 mutants, providing a strong indication that HIP-55 also regulates the endocytic machinery in T cells. Surprisingly, this effect was rather specific, because the expression of the costimulatory receptor CD28 was slightly increased, whereas the expression of the glycosylphosphatidylinositol-linked protein CD59 was unaffected. Glycosylphosphatidylinositol-linked proteins and antigen receptor complexes utilize different internalization pathways (41, 42), suggesting that HIP-55 is specifically involved in clathrin-coated vesicle trafficking. Supporting this notion is our observation that expression of HIP-55 in kidney cells is required during transferrin receptor endocytosis (25). In non-hematopoietic cells, HIP-55 interacts with dynamin, a GTPase involved in the clathrin-mediated endocytic pathway (24, 25). In yeast, where the endocytic process and the actin cytoskeleton have been linked by genetic studies, Abp1 clearly functions in receptor endocytosis (17, 40). We have observed here an important reduction of CD3 expression on T cells overexpressing wild-type HIP-55 but not ADF-H and SH3 mutants, providing a strong indication that HIP-55 also regulates the endocytic machinery in T cells. Surprisingly, this effect was rather specific, because the expression of the costimulatory receptor CD28 was slightly increased, whereas the expression of the glycosylphosphatidylinositol-linked protein CD59 was unaffected. Glycosylphosphatidylinositol-linked proteins and antigen receptor complexes utilize different internalization pathways (41, 42), suggesting that HIP-55 is specifically involved in clathrin-coated vesicle trafficking. Supporting this notion is our observation that expression of HIP-55 in kidney cells is required during transferrin receptor endocytosis (25). In non-hematopoietic cells, HIP-55 interacts with dynamin, a GTPase involved in the clathrin-mediated endocytic pathway (24, 25). We have observed here an important reduction of CD3 expression on T cells overexpressing wild-type HIP-55 but not ADF-H and SH3 mutants, providing a strong indication that HIP-55 also regulates the endocytic machinery in T cells. Surprisingly, this effect was rather specific, because the expression of the costimulatory receptor CD28 was slightly increased, whereas the expression of the glycosylphosphatidylinositol-linked protein CD59 was unaffected. Glycosylphosphatidylinositol-linked proteins and antigen receptor complexes utilize different internalization pathways (41, 42), suggesting that HIP-55 is specifically involved in clathrin-coated vesicle trafficking. Supporting this notion is our observation that expression of HIP-55 in kidney cells is required during transferrin receptor endocytosis (25). In non-hematopoietic cells, HIP-55 interacts with dynamin, a GTPase involved in the clathrin-mediated endocytic pathway (24, 25).

Fig. 7. Effect of HIP-55 expression on ligand-induced TCR/CD3 down-modulation. A, HIP-55 reduced surface expression of CD3. Mixed population was either left unstimulated or stimulated with anti-CD3 antibody for 1 h. After washes, cells were stained with a phycoerythrin secondary antibody and analyzed by flow cytometry. B, expression of overexpressed and endogenous proteins was analyzed by anti-V5 and anti-HIP-55 immunoblots. C, histograms displaying the percentage of CD3 expressed observed in three independent experiments for both unstimulated and stimulated conditions.

S. Mise-Omata, unpublished observations.
The expression of some components of T cell signaling has recently provided evidence of a connection between TCR trafficking and responsiveness to antigenic stimulation. For example, overexpression of Src-like adaptor protein-2 (SLAP-2) in Jurkat cells reduced CD3-TCR complex expression, an event likely involved in the diminished NFAT activation observed in these cells (43). Interestingly, SLAP-2 co-localized with endosomes in thymocytes (44). Reduced trafficking of activated TCR in lymphocytes from C-B1 and Cb-b double knock-out mice has been implicated in T cell hyperresponsiveness to anti-CD3 stimulation (45) and inactivation of the adaptor CD2AP, which affects TCR endocytosis and leads to IL-2 hyperproduction following antigenic stimulation (46). These observations are reminiscent of our data showing that HIP-55 and/or HPK1-null Jurkat cells are hyperresponsive to anti-CD3 stimulation. It is very important to note here that, in our transient transfections assays used to examine the role of the HIP-55-HPK1 complex during TCR-induced gene activation, TCR expression was almost unaffected as judged by FACS analysis (data not shown), suggesting that the two processes in our study that implicate HIP-55 are overlapping but distinct. Nevertheless, our data raise a important question. Endocytosis is known to be regulated by serine/threonine phosphorylation of proteins associated with clathrin-coated vesicles. However, the identities of the involved kinases have remained elusive. The GCK-like kinase GLK interacts with endophilin (47), suggesting that GCK family members, including HPK1, might be involved in the regulation of clathrin-mediated endocytosis. It will therefore be useful to examine how endocytosis and the expression of TCRs are affected in HIP-55 and/or HPK1-null lymphocytes or animals.

Sustained TCR-major histocompatibility complex (MHC) interactions and dynamic reorganization of the actin cytoskeleton are required for optimal delivery of T cell activation signals. These events take place at the T cell-APC contact site within a highly organized structure called the IS. It has become clear from genetic and biochemical studies that one critical function of the IS is probably TCR signaling down-regulation and endocytosis. We would like to propose that, by regulating both gene activation and TCR expression, the actin-binding protein HIP-55 may allow signal integration between the IS, the actin activation and TCR expression, the actin-binding protein cytosis. We would like to propose that, by regulating both gene of the IS is probably TCR signaling down-regulation and endocytosis. It has become clear that interactions and dynamic reorganization of the actin cytoskeleton are required for optimal delivery of T cell activation signals.

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