Docking Protein Gab2 Is Phosphorylated by ZAP-70 and Negatively Regulates T Cell Receptor Signaling by Recruitment of Inhibitory Molecules*

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To maintain various T cell responses and immune equilibrium, activation signals triggered by T cell antigen receptor (TCR) must be regulated by inhibitory signals. Gab2, an adaptor protein of the insulin receptor substrate-1 family, has been shown to be involved in the downstream signaling from cytokine receptors. We investigated the functional role of Gab2 in TCR-mediated signal transduction. Gab2 was phosphorylated by ZAP-70 and co-purified with phosphoproteins, such as ZAP-70, LAT, and CD3ε, upon TCR stimulation. Overexpression of Gab2 in Jurkat cells or antigen-specific T cell hybridomas resulted in the inhibition of NF-AT activation, interleukin-2 production, and tyrosine phosphorylation. The structure-function relationship of Gab2 was analyzed by mutants of Gab2. The Gab2 mutants lacking SHP-2-binding sites mostly abrogated the inhibitory activity of Gab2, but its inhibitory function was restored by fusing to active SHP-2 as a chimeric protein. A mutant with defective phosphatidylinositol 3-kinase binding capacity also impaired the inhibitory activity, and the pleckstrin homology domain-deletion mutant revealed a crucial function of the pleckstrin homology domain for localization to the plasma membrane. These results suggest that Gab2 is a substrate of ZAP-70 and functions as a switch molecule toward inhibition of TCR signal transduction by mediating the recruitment of inhibitory molecules to the TCR signaling complex.

T cells recognize antigens (Ag) presented by the major histocompatibility complex on antigen-presenting cells (APC). An Ag recognition signal is then transmitted to the cytoplasm via the T cell antigen receptor (TCR) complex and induces T cell activation, leading to various cellular events such as proliferation, apoptosis, anergy, and differentiation as well as a variety of effector functions. To evoke appropriate T cell responses and maintain immune equilibrium, these stimulatory signals must be regulated by negative signals delivered via inhibitory molecules (1). In contrast to positive regulation, however, the precise mechanism of negative regulation of T cells has not as yet been widely characterized. In B cells and NK cells, several inhibitory receptors containing immunoreceptor tyrosine-based inhibitory motif such as FcRRIIB, CD22, CD72, killer inhibitory receptor, and Ly-49 have been reported (2). Upon stimulation by ligand binding, these inhibitory receptors become tyrosine-phosphorylated and recruit Src homology 2 domain-containing tyrosine phosphatases (SHP-1 and SHP-2) and/or Src homology 2 domain-containing inositol-5-phosphatase, resulting in the inhibition of antigen receptor-mediated effector function and cytokine expression. In T cells, it has been well established that CTLA-4 and PD-1 are induced upon T cell activation and function as an inhibitory co-receptor at a late activation phase (3, 4). However, there has been little investigation of the inhibitory machineries through typical inhibitory receptors in T cells, suggesting the possibility that another inhibitory mechanism downstream of the “multitask” TCR itself may play a role in negative regulation.

The engagement of TCR triggers activation of protein-tyrosine kinases and their downstream signaling events such as the increase of intracellular Ca2+ and activation of Ras (5, 6). One of the initial events is the tyrosine phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) of the CD3 chains by Src family kinases, Lck and Fyn. Following the phosphorylation of ITAM, ZAP-70 is recruited via its SH2 domains and activated, and then it mediates further downstream phosphorylation (7). Recently, two adaptor proteins, LAT and SLP-76, were identified as substrates of ZAP-70, and both were revealed to play crucial roles in activating Ras and phospholipase C-γ1-Ca2+ signaling pathways (8). In contrast to the extensive analysis of activation signals through ITAM, ZAP-70, and adaptors, the regulatory mechanism by inhibitory signals has not been clearly understood.

SHP-2, an SH2-containing protein-tyrosine phosphatase (SH-PTP2/PTP1D/Syp), is expressed ubiquitously, and it is known to function as a positive signaling element in multiple receptor signaling pathways (9–11). However, recent studies have also demonstrated the negative role of SHP-2 in several receptor-signaling pathways in hematopoietic cells (12–14).
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Intrinsin receptor (IRS)-related Gab2 (Grb2-associated binder) family adaptor proteins were recently identified as SHP-2-binding molecules (15). Gab2 is a member of the Gab family of adaptor molecules consisting of Gab1, Gab2, and Drosophila Daughter of Sevenless. Both Gab1 and Gab2 contain a pleckstrin homology (PH) domain in the amino-terminal region as well as a tyrosine-based motif and proline-rich sequences, which are potential binding sites for various SH2 domains and SH3 domains, respectively (16–19). The Gab family functions as scaffold proteins by interacting with multiple signaling molecules, such as SHP-2, p85 phosphatidylinositol (PI) 3-kinase, phospholipase C-γ1, and Grb2, and is involved in the expression of biological activities through a variety of growth factors and cytokines. Both Gab1 and Gab2 are tyrosine-phosphorylated upon stimulation of T and B cell antigen receptors as well as receptors for growth factors and cytokines (20–22). Because Gab2 is preferentially phosphorylated upon TCR stimulation (16, 18), we investigated the function of Gab2 in the TCR signal transduction pathway.

In the present study, we report that Gab2 is a substrate of ZAP-70 and is associated with the TCR signaling complex upon T cell activation, and further that Gab2 mediates inhibitory function via recruitment of inhibitory molecules, particularly SHP-2.

EXPERIMENTAL PROCEDURES

Cells and Reagents—A keyhole limpet hemocyanin-specific and I-A-restricted murine Th1-type T cell clone, 25-1-8 (23), was maintained in RPMI 1640 supplemented with 10% fetal calf serum and 5 μg/ml murine IL-2. These T cells were stimulated with keyhole limpet hemocyanin (100 μg/ml, Calbiochem) and irradiated C3H/HeJ spleen cells every 3–4 weeks. Jurkat T cells and pigeon cytochrome c-specific T cell hybridoma, 2B4, were maintained in 10% fetal calf serum supplemented RPMI 1640. Stable transfectants derived from Jurkat cells were established by selection in the culture medium with 4 mg/ml of G418. 293T cells and a retrovirus packaging cell line, phoenix, kindly provided by Dr. M. Iwashima, Medical College of Georgia) were established by stable transfection of the ecotropic retrovirus vector into Jurkat T cells. A moth cytochrome c peptide analogue, DASP, was synthesized and used as Ag.

Antibodies—Abs used for TCR stimulation of Jurkat cells were C305, an anti-TCR mAb (24) (kindly provided by Dr. A. Weiss, University of California, San Francisco), and OKT3, an anti-CD3 mAb. The Abs used for blots and immunoprecipitation are as follows: anti-FLAG M2 mAb (Sigma); anti-CID3 mAbs, 6B10.2 (Santa Cruz Biotechnology); hamster anti-CID3 serine, H146 (provided by Dr. R. Kubo, BD Biosciences); rabbit anti-LAT serum (25) (provided by Dr. L. Samelson, National Institutes of Health); sheep anti-SLP-76 serum (26) (provided by Dr. G. Koretsky, University of Iowa); anti-ZAP-70 mAb, 2F3.2 (7) (provided by Dr. Y. Koga, Kyushu University); 4G10 and rabbit anti-SHP-1 serum (Upstate Biotechnology, Inc.); rabbit anti-Erk2 mAb (Cell Signaling Technology); rabbit anti-Grb2, represented as pp95 

RESULTS

Gab2 is Tyrosine-phosphorylated and Associates with Phosphoproteins Upon TCR Stimulation—As the first step in analyzing the function of Gab2, we investigated the phosphorylation of Gab2 following TCR ligation. Jurkat T cells were stimulated with anti-CD3 Ab, and the cell lysates were immunoprecipitated with anti-Gab2 Ab, followed by blotting with anti-PY mAb. As shown in Fig. 1A, Gab2, represented as pp95–100, was tyrosine-phosphorylated upon stimulation and associated with several phosphoproteins such as pp70, pp96, pp21, and pp18 in Jurkat T cells (Fig. 1A, lane 6). Consistent with previous studies (16, 18) that Gab2 binds to SHP-2 upon TCR ligation, independent blots of the same samples with anti-SHP-2 Ab revealed that pp70 contains SHP-2 (Fig. 1A, lane 8). Another SH2-containing phosphatase, SHP-1, predominantly expressed in hematopoietic cells, was not detected in the anti-Gab2 immunoprecipitates (data not shown). To identify the phosphoproteins co-precipitated with Gab2, anti-Gab2 precipitates were blotted with several Abs as shown in Fig. 1A. Independent blots suggested that pp21 and pp18 contain the CD3ζ chain. Furthermore, pp70 appeared to correspond not.
To identify the tyrosine kinase responsible for these observations suggest that Gab2 is involved in the TCR signaling complex in Jurkat cells. Jurkat phosphorylation and assembling with the TCR upon TCR stimulation.

Shp-2, ZAP-70, rabbit Ig, and human CD3ε (lanes 7–10). Total lysates were also blotted with anti-FY as a control (lanes 1 and 2). The positions of the molecular mass standards are indicated in the left margin. B, Gab2 phosphorylation and associated proteins in mouse splenic T cells. CD4+ and CD8+ T cells were purified from splenocytes as described above. Cell lysates from unstimulated and anti-CD3-stimulated splenic T cells were immunoprecipitated with rabbit IgG (control Ig) and anti-Gab2 Ab. Each precipitate was blotted with anti-FY Ab (upper panel). The membrane was rebotted with anti-Gab2 Abs as a control (lower panel). IP, immunoprecipitation.

Gab2 is Tyrosine-phosphorylated by ZAP-70 and Associated with SHP-2—To identify the tyrosine kinase responsible for the phosphorylation of Gab2 following TCR triggering, 293T cells were co-transfected with FLAG-tagged Gab2 and ZAP-70 and/or Lck as possible candidates. Gab2 was strongly phosphorylated when co-expressed with ZAP-70 and Lck (Fig. 2A, top panel, lane 3). However, Lck alone did not induce association of SHP-2 and Gab2, although weak phosphorylation of Gab2 did occur (Fig. 2A, lane 2). The Gab2-SHP-2 complex could not be detected even in cells expressing Lck together with a kinase-inactive ZAP-70 (data not shown), indicating that the formation of this complex was dependent on ZAP-70 kinase activity. A Gab2 mutant containing a phenylalanine substitution of Tyr-614, one of the potential SHP-2 binding sites, was constructed and introduced into 293T cells with ZAP-70 and/or Lck. Gab2(Y614F) was tyrosine-phosphorylated at a similar level to Gab2(WT), whereas significant interaction of Gab2(Y614F) with SHP-2 was not detected (Fig. 2A, lane 6), consistent with recent report (35) concerning a role of the tandem tyrosine motif in Gab1-SHP-2 interaction. These data demonstrate that Tyr-614 is phosphorylated by ZAP-70 and serves as a major binding site for SHP-2. It has been shown that the SH2 domains of SHP-2 are responsible for the association with Gab family proteins (36, 37). Fig. 2B supports this model, because overexpression of the SH2 domains of SHP-2 (SHP-2-ΔPTP) blocked the binding of endogenous SHP-2 to phosphorylated Gab2 in a competitive manner.

We further examined Gab2 tyrosine phosphorylation in wild-type and ZAP-70-deficient Jurkat (P116) cells. Compared with Jurkat cells, Gab2 phosphorylation following TCR ligation was significantly decreased in P116 cells (Fig. 2C, lane 4), despite the fact that Lck expression in P116 cells is normal (38). Reblotting with anti-Gab2 Ab indicated that equal amounts of Gab2 were immunoprecipitated (Fig. 2C, lower panel).

These results demonstrate that ZAP-70 is responsible for TCR-induced Gab2 phosphorylation. Because it has been suggested that other kinases are involved in the phosphorylation of Gab2 upon cytokine stimulation (18, 39), the phosphorylation of Gab2 by various protein-tyrosine kinases can be expected to depend on the type of receptor triggered.

SHP-2-binding Sites Are Critical for the Inhibitory Function of Gab2—The function of Gab2 in TCR-mediated signal transduction was investigated by analyzing the effects of Gab2 overexpression on NF-AT activation. As shown in Fig. 3A, Gab2 significantly suppressed NF-AT-luciferase activity upon stimulation with immobilized anti-TCR mAb in a dose-dependent manner. To analyze the mechanism of Gab2-mediated inhibition of T cell activation, the functions of Gab2(WT) and Gab2(Y614F), the latter of which lacks the major SHP-2-binding site in T cell activation, were examined by transient assay. As shown in Fig. 3B, Gab2(WT)-transfected cells showed significant reduction of IL-2 production as compared with the mock-transfected cells, whereas such a strong inhibitory effect was not observed in Gab2(Y614F)-expressing cells.

To investigate further whether Gab2 inhibits Ag-specific cytokine production, Gab2(WT) and Gab2(Y614F) were introduced into an Ag-specific mouse T cell hybridoma, 2B4, by retrovirus-mediated gene transfer using a vector containing

Fig. 1. Gab2 is tyrosine-phosphorylated and is associated with SHP-2 upon TCR stimulation. A, Gab2 phosphorylation and assembling with the TCR signaling complex in Jurkat cells. Jurkat cells (7 × 10⁶) were stimulated by cross-linking with anti-CD3 mAb (OKT3) and sheep anti-mouse Ig for 2 min or left unstimulated, and the cell lysates were immunoprecipitated with normal rabbit IgG (control Ig) or anti-Gab2 Ab and analyzed by Western blotting with anti-FY Ab (lanes 3–6). An aliquot of each sample was also blotted with Abs against Gab2, SHP-2, ZAP-70, rabbit Ig, and human CD3ε (lanes 7–10). Total lysates were also blotted with anti-FY as a control (lanes 1 and 2). The positions of the molecular mass standards are indicated in the left margin. B, Gab2 phosphorylation and associated proteins in mouse splenic T cells. CD4+ and CD8+ T cells were purified from splenocytes as described above. Cell lysates from unstimulated and anti-CD3-stimulated splenic T cells were immunoprecipitated with rabbit IgG (control Ig) and anti-Gab2 Ab. Each precipitate was blotted with anti-FY Ab (upper panel). The membrane was rebotted with anti-Gab2 Abs as a control (lower panel). IP, immunoprecipitation.
The combination of PMA plus Ca\(^{2+}\) exhibit full inhibitory activity. Stimulation of these cells with infected cells, whereas Gab2(Y614F)-expressing cells failed to exhibit the proximal events of the TCR signaling pathway. These results indicate that Tyr-614, a major SHP-2-binding site, is involved in Gab2-mediated suppression of T cell activation.

SHP-2 is reportedly recruited to several inhibitory receptors and mediates inhibitory activity following ligand engagement (13, 14, 41, 42). On the other hand, a receptor-like PTPase, CD148, has been shown to inhibit TCR signaling by dephosphorylating proximal signaling molecules such as phospholipase C-\(\gamma\)1 and LAT (43). Taken together, our observations raise the possibility that Gab2-associated SHP-2 may inhibit the TCR signaling pathway by dephosphorylating proximal signaling molecules. To examine the phosphorylation status in detail, we established Jurkat cells expressing WT and Y614F Gab2 by retroviral infection and analyzed bulk populations to avoid clonal variation. To infect an ecotropic retrovirus into Jurkat cells, we established a Jurkat transfectant stably expressing the ectropic viral receptor, J.EcoR. By using this system, we introduced Gab2(WT) construct, Gab2(Y614F) construct, or vector alone (Mock) into J.EcoR, and the GFP bulk population was sorted as described previously (3). Similar levels of Gab2 protein were expressed in these infected lines (Fig. 3D, lower panel). Because the CD3\(\varepsilon\) chain has been reported to be a substrate of SHP-2 in T cells (12), we examined the phosphorylation levels of CD3\(\varepsilon\) in these lines after TCR engagement by immunoprecipitation with anti-\(\varepsilon\) mAb, followed by blotting with anti-PY mAb. As shown in Fig. 3D, the phosphorylation levels of the \(\varepsilon\) chain were attenuated by Gab2(WT) expression but not by Gab2(Y614F). Therefore, the inhibitory function of Gab2 is mediated, at least in part, through SHP-2-dependent dephosphorylation of the \(\varepsilon\) chain.

We further tested the synergistic contribution of Tyr-643, the other potential SHP-2-binding site, for T cell activation. To confirm the SHP-2 binding of these mutants in T cells, transfected Gab2 was immunoprecipitated and blotted with anti-SHP-2 following TCR stimulation. Fig. 4A showed that SHP-2 is associated with Gab2 in a stimulation-dependent manner (lane 4). Whereas faint SHP-2 binding (3% of wild type) was detected with Y614F, no SHP-2 interacted with Gab2(Y614F)/Y643F (Fig. 4A, lanes 6 and 8). Reblotting with anti-Gab2 confirmed that similar amounts of Gab2 mutants were expressed and immunoprecipitated (Fig. 4A, middle panel). Stimulation-dependent association with P13 kinase, one of the major binding molecules, was not affected by these mutations (Fig. 4A, as reported previously (44). Total lysates were also blotted with anti-SHP-2 or anti-P13 kinase Abs as a control. Because \(\varepsilon\) phosphorylation by Lck serves the binding sites for SHP-2 following TCR stimulation (45), we suspected that Gab2 expression also reduces the levels of cellular tyrosine phosphoproteins (Fig. 4B). Indeed, the levels of total tyrosine phosphorylation upon TCR stimulation were attenuated in Gab2(WT), whereas Y614F and Y614F/Y643F mutants restored the level (Fig. 4B). The suppressive activity of Gab2 in total phosphorylation appears to correlate with the SHP-2 binding ability.

Next, to analyze the effect of these mutants on gene expressions, the induction of CD69 expression on each transfectant upon TCR stimulation was compared. Consistent with tyrosine phosphorylation, the inhibition of the cell surface expression of CD69 was correlated with SHP-2 binding (Fig. 4C, left panel). Used as a control, PMA induced similar levels of CD69 expression in these cell lines (Fig. 4C, right panel). Thus, Tyr-614 and Tyr-643 of Gab2 are required for TCR-dependent CD69 expression. Because \(\varepsilon\) phosphorylation by Lck is an initial trigger of downstream cascades (7), these observations support our hypothesis that Gab2-associated SHP-2 becomes active and dephosphorylates the CD3\(\varepsilon\) chain, resulting in efficient attenuation of TCR signaling.
Gab2 Is Associated with LAT in a Tyrosine Phosphorylation-dependent Manner—The next step examined the molecular basis for the association of Gab2 within the TCR signaling complex by identifying a target molecule within the complex using a heterologous expression system. We failed to detect any obvious interaction of Gab2 with CD3ζ and ZAP-70 in 293T cells even in the presence of Lck and/or ZAP-70 (data not shown). However, LAT, known as a scaffold protein in T cells, was found to bind to Gab2 when co-expressed with Lck and ZAP-70 (Fig. 5A, lane 4). This interaction was significant because the relative ratio of phosphorylated LAT to phosphorylated Gab2 in Gab2 immunoprecipitates was similar to that in total cell lysates (Fig. 6A, lanes 4 and 8). Y614F/Y643F mutations of Gab2 did not affect the association with LAT, indicating that tyrosine 614/643 or SHP-2 binding is not required for the LAT-Gab2 complex formation (Fig. 5B, lane 4). As was seen in normal T cells (Fig. 1B), these results suggest that Gab2 interacts with LAT upon TCR stimulation. The molecular mechanism of this association is discussed below.

Expression of a Chimeric Protein Consisting of SHP-2 Binding-defective Gab2 and Active SHP-2 Results in Inhibition of TCR Signaling—Although our data indicated that SHP-2-binding sites of Gab2 are involved in the inhibitory function of Gab2, the possibility still remains that other molecules that bind to phosphorylated Tyr-614 and/or Tyr-643 are responsible for the inhibitory function of Gab2. Alternatively, these tyrosine residues may contribute to phosphorylation of other sites that could mediate the suppressive effect. However, the latter seems less likely because the phosphorylation level of Gab2(Y614F/Y643F) was similar to that of Gab2(WT) after TCR ligation. To address this question, we constructed a chimeric molecule in which the active form of SHP-2 (SHP-2ΔSH2) is fused to the carboxyl terminus of Gab2(Y614F/Y643F) lacking a SHP-2-binding site (G/S chimera) as illustrated in Fig. 6A. 293T cells were transiently transfected with expression constructs encoding either Gab2(Y614F/Y643F)/SHP-2 chimera(WT) or its catalytically inactive substrate. As shown in Fig. 6C, wild type but not C/A chimeratransfected cells exhibited significant PTPase activity, demonstrating that Gab2(Y614F/Y643F)/SHP-2 chimera has constitutive PTPase activity. These chimeric cDNAs were then introduced into J.EcoR cells, and GFP+ cells were sorted and stimulated with super Ag SEE. Although Gab2(Y614F/Y643F) lost inhibitory activity for the induction of the cell surface expression of CD69 (Fig. 4C), Gab2(Y614F/Y643F)/SHP-2 chimera exhibited the inhibitory activity upon stimulation with
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FIG. 4. SHP-2-binding sites of Gab2 are critical for the suppression of tyrosine phosphorylation and CD69 expression following TCR ligation. A, the association of SHP-2 with Gab2 mutants upon TCR stimulation. J.EcoR cells were infected with vector alone (Mock) or expression constructs for Gab2 WT, Y614F, and Y614F/Y643F using pMX-IRES-GFP retrovirus vector. GFP+ cells were sorted (>98% purity) and stimulated with anti-CD3 mAb for 2 min. Cell lysates were immunoprecipitated (IP) with anti-FLAG Ab and blotted with anti-SHP-2, anti-PI3 kinase, or anti-Gab2 Abs. Total lysates were also blotted for SHP-2 or PI3 kinase as a control (lower panels). B, total cellular tyrosine phosphorylation in Gab2-expressing cells. Mock, Gab2(WT), Gab2(Y614F), and Gab2(Y614F/Y643F)-expressing cells were stimulated by anti-CD3 mAb for 2 min. 10 μg of protein of total cell lysates was analyzed by Western blotting with anti-PY and anti-ZAP-70 Abs. C, function of Gab2 mutants on TCR-stimulated induction of CD69 expression. Gab2 transfectants described in B were left unstimulated (open columns) or stimulated by anti-CD3 mAb (closed columns) for 24 h, and the expression of CD69 was analyzed with phycoerythrin-labeled anti-CD69 mAb by flow cytometry (left panel). CD69 expressions upon stimulation with PMA plus A23187 were shown as controls (right panel). The data were expressed as the mean fluorescence intensity (MFI) ± S.D. The expression level of Gab2 in each transfectant is shown in A.

SEE (Fig. 6D). In contrast, the chimera containing SHP-2.C/A failed to inhibit the response. These results suggest that physically associated active SHP-2 is responsible for the Gab2-mediated inhibitory activity, and it is unlikely that other molecules binding to these sites mediate inhibition.

Multiple Domains Are Required for the Inhibitory Function of Gab2—It has recently been reported that Gab2-mediated inhibition of TCR signaling requires PI3 kinase-binding sites (44). We also analyzed these domains for Gab2 function. Consistent with the previous reports, when J.EcoR cells were infected with Gab2(Y452F/Y476F/Y584F;Y3F) mutant which completely abolishes the PI3 kinase binding (Fig. 7, B and C), the mutant partially restored IL-2 response (Fig. 7A). This result suggests the involvement of PI3 kinase in Gab2-induced suppression. However, we found that the phosphorylation level and SHP-2 binding ability of Gab2(Y3F) were also attenuated to 26% of those of Gab2(WT) in 293T cells (Fig. 7B, lane 4). Consistent with this, 70% of the reduction of SHP-2 binding was observed in T cells upon TCR stimulation (Fig. 7C, lane 6). Thus, in addition to the loss of PI3 kinase binding, the restoration from the inhibition of IL-2 production in Gab2(Y3F) can be attributed partly to the reduced level of SHP-2 binding. It has also been suggested that the PH domain of Gab2 is required for inhibitory functions possibly by mediating the translocation of Gab2 to the plasma membrane (44). Analysis of the PH deletion mutant of Gab2 (Gab2/PH) in our system provides support for this concept, as Gab2(ΔPH) mutant did not translocate to the membrane, was localized in the cytoplasm (Fig. 7C), and failed to show inhibition of SEE-induced IL-2 production (Fig. 7A).

Collectively, the scaffold protein Gab2 seems to block effectively TCR signaling by recruiting several binding proteins, SHP-2, PI3 kinase, and/or other inhibitory molecules, to the plasma membrane.
In the present study, we found that Gab2 is phosphorylated by ZAP-70, associates with the TCR signaling complex, and acts as an inhibitory adaptor molecule via recruitment of SHP-2 following TCR ligation. In contrast to other members of IRS-1 family adaptors, the Gab family is tyrosine-phosphorylated and forms a complex with SHP-2 upon stimulation through Ag receptors as well as receptors for growth factors and cytokines (16, 18). Notably, Gab2 is heavily tyrosine-phosphorylated upon TCR stimulation compared with Gab1 (18). Tyrosine phosphorylation was observed not only in Jurkat cells but also in splenic T cells upon TCR stimulation. Furthermore, Gab2 was also associated with several phosphoproteins such as ZAP-70, LAT, and CD3ζ in a stimulation-dependent manner. Together with a recent report by Ingham et al. (37) that Gab1 is phosphorylated and associated with various phosphoproteins following B-cell receptor stimulation, our data also indicate that the Gab family can function as a scaffold protein downstream of Ag receptors.

The structural basis of the association of Gab2 within the Ag receptor complexes is yet unclear. However, our finding that Gab2 associates with LAT in vivo in a phosphorylation-dependent manner may provide the molecular basis for the involvement of Gab2 within the TCR signaling complex. Because LAT lacks any phosphotyrosine binding domains, Gab2-LAT binding might be mediated by the direct interaction of Gab2 itself or, alternatively, through SH2-containing adaptors to the phosphorylated LAT. Indeed, we found that Gab2-LAT interaction is mediated by Gads/Grb2 adaptor molecule. In addition, the PH domain may contribute to the membrane localization of Gab2 to form a stable complex with other TCR-related signaling molecules. Gab1 has been reported to localize in the plasma membrane through the binding of its PH domain and phosphatidylinositol 1,4,5-trisphosphate in fibroblasts or B cells upon several stimuli, where it can be phosphorylated and participate in receptor signaling (45–47). Similarly, Gab2 may be recruited to the plasma membrane via its PH domain upon T cell activation, which greatly enhances the accessibility to membrane-associated molecules such as LAT or the TCR components. Accordingly, PH domain-deleted mutant Gab2 (Gab2ΔPH) did not localize to the plasma membrane or exert an inhibitory effect on IL-2 production in Jurkat cells. The reason for Gab2 to be localized to the plasma membrane without TCR stimulation may depend on the reported elevated basal level of phosphatidylinositol 1,4,5-trisphosphate in Jurkat cells (48).

Because we have previously shown that ZAP-70 becomes catalytically active when phosphorylated by Lck (49), Lck and ZAP-70 were co-expressed with Gab2 to analyze the phosphorylation of Gab2. Gab2 was heavily phosphorylated and associated with SHP-2 only in the presence of Lck-activated ZAP-70, but not in the presence of kinase-inactive ZAP-70 mutant. Thus, Gab2 could be phosphorylated by ZAP-70 and create major binding sites for the SH2 domain of SHP-2. Consistent with this, Gab2 phosphorylation was not detected in ZAP-70-deficient Jurkat cells, P116. This is foremost evidence that suggests that both positive and negative signals are branched from ZAP-70 through phosphorylation of different substrates. Understanding the mechanisms of how the phosphorylation of positive signal-inducing substrates (SLP-76 and LAT) or negative signal-inducing substrates (Gab2) could be regulated may eventually lead to the ability to possibly manipulate the regulation of T cell responses.

Overexpression of Gab2 in T cells resulted in down-regulation of TCR-mediated NF-AT activation and IL-2 production. It is likely that this inhibitory function of Gab2 is mostly mediated by SHP-2, because the Gab2 Y614F and Y614F/Y643F mutants could not bind to SHP-2 or mediate inhibitory function. However, the possibility still remains that Tyr-614 and/or Tyr-643 of Gab2 may recruit other molecules responsible for inhibition. Nevertheless, our observation that the loss of inhibitory function of SHP-2 binding-defective Gab2 was rescued by attaching active SHP-2 to the carboxyl terminus suggests that the inhibitory function of Gab2 is largely dependent on SHP-2. The fact that the overexpressed Gab2 could mediate the inhibitory effect with endogenous SHP-2 suggests that the level of phosphorylated Gab2 regulates the inhibitory activity.

The crystal structure of SHP-2 reveals a "closed" domain arrangement in which the two SH2 domains form a contour around the phosphatase domain in the absence of any phosphorylated binding partners. Upon binding with a tyrosine-phosphorylated partner protein through its SH2 domain,
SHP-2 becomes catalytically active and dephosphorylates its substrates (50). Deletion of the SH2 domain of SHP-2 results in constitutive PTPase activation (35). It has been reported that CTLA-4-bound SHP-2 efficiently dephosphorylates Shc and the ζ chain in T cells (12, 51). It appears likely that, in the same manner, Gab2-associated SHP-2 becomes catalytically active and dephosphorylates the ζ chain, which leads to decreased downstream signals. Fearnson and Alexander (11) reported that the overexpression of PTPase-inactive SHP-2 decreases TCR signaling. These apparently contradictory observations could be partially explained by the multiple roles of SHP-2 via its multiple binding targets in T cells. SHP-2 may have both positive and negative functions in T cells, and the negative function might be largely dependent on Gab2.

Although Gab family proteins have been reported as positive signal transducers in many types of cells, we and others (44) have revealed that Gab2 is a negative regulator in TCR signaling. Furthermore, Dok, a Gab2-related PH domain-containing adaptor molecule, has recently been shown to function as a negative regulator in B-cell receptor signaling (45-47). Although it had been suggested to play a positive role in cell growth (52), Dok2 and Dok3 have also been reported to inhibit lymphocyte activation by recruiting Rack1 and Csk, respectively (53, 54). Gab2 might be a member of the "PH domain-containing inhibitory adaptors." Because Gab2 is known to interact with multiple signaling molecules such as Crk and PI3 kinase, besides SHP-2, such proteins may also contribute to the inhibitory function of Gab2. Recently, it was reported that Gab2 inhibits TCR-induced NF-AT activation via the recruiting of PI3 kinase but not SHP-2 (44). Indeed, we also confirmed that PI3 kinase-binding defective Gab2 showed impaired inhibitory activity, although a part of this impairment might be due to the decreased SHP-2 binding ability of Gab2(Y452F/Y476F/ Y584F; Y3F) (Fig. 7). As for the involvement of SHP-2, it is reported that the SHP-2-binding defective mutant still has an inhibitory effect on IL-2 promoter activity induced by PMA plus anti-CD3 (44). At present, the reason for this apparent discrepancy is unclear. However, the difference might be due to experimental differences such as the fact that we utilized anti-TCR stimulation in the absence of PMA, or the more physiological super Ag plus APC. Furthermore, we measured IL-2 production and CD69 induction by stable transfectant as well as promoter analysis by transient transfection upon stimulation to evaluate the function of various Gab2 mutants. Taken together, immune receptors may have conferred an inhibitory function on IRS-1-related multiple adaptor molecules in a lymphocyte-specific manner to evoke appropriate immune responses to small amounts of antigens.

It is also possible that Gab2 functions as a negative feedback regulator, based on the observation that Gab2 is significantly up-regulated on T cell activation, as shown recently (39). Cytokine-inducible SH2 protein/suppressor of cytokine signaling family molecules are also induced after cytokine stimulation and inhibit their signaling. Cytokine-inducible SH2 protein/suppressor of cytokine signaling family proteins play an essential role in the maintenance of immunological homeostasis as a negative feedback loop (55). By an analogy with the cytokine-inducible SH2 protein/suppressor of cytokine signaling family, our present data suggest that Gab2 may function as...
a negative feedback loop of TCR signaling. The physiological roles of Gab2 in the whole immune response are now under investigation.

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