Activation of RasGRP3 by phosphorylation of Thr-133 is required for B cell receptor-mediated Ras activation

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The Ras signaling pathway plays a critical role in B lymphocyte development and activation, but its activation mechanism has not been well understood. At least one mode of Ras regulation in B cells involves a Ras-guanyl nucleotide exchange factor, RasGRP3. We demonstrate here that RasGRP3 undergoes phosphorylation at Thr-133 upon B cell receptor cross-linking, thereby resulting in its activation. Deletion of phospholipase C-γ2 or pharmacological interference with conventional PKCs resulted in marked reduction in both Thr-133 phosphorylation and Ras activation. Moreover, mutation of Thr-133 in RasGRP3 alone severely impaired its ability to activate Ras in B cell receptor signaling. Hence, our data suggest that PKC, after being activated by diacylglycerol, phosphorylates RasGRP3, thereby contributing to its full activation.

The Ras pathway has been implicated in supporting survival and differentiation of pre-B cells as well as mature B cells. Indeed, introduction of a constitutive active form of Ras into a Rag-null background can cause progression of pro-B cells to pre-B and subsequent mature B cells (1, 2). Conversely, expression of a dominant negative form of Ras markedly reduces the number of pre-B cells and immature B cells (3, 4). These findings, given the importance of pre-B cell receptor (pre-BCR) and BCR in B cell survival and differentiation (5–8), suggest a crucial role for Ras in pre-BCR- and BCR-mediated cell fate decision.

There is a good relationship between diacylglycerol (DAG), a product of phospholipase C (PLC)-γ, and Ras activation in lymphocytes, as shown by findings that phorbol ester stimulation results in accumulation of active GTP-bound Ras (9). Further strengthening this relationship, the deletion of PLC-γ2 causes impaired BCR-mediated Ras activation (10). Because RasGRP, a member of the cdc25 family of Ras guanyl nucleotide exchange factors (Ras-GEFs) (11), has a DAG-binding C1 domain, DAG generated upon antigen receptor stimulation is thought to contribute to recruiting RasGRP to the membrane, where it interacts with Ras. Indeed, in B cells, a membrane-attached form of RasGRP3 can rescue the defective Ras activation to some extent, but not completely, in PLC-γ2-deficient DT40 B cells (10). Thus, these data suggest that the recruitment mechanism is necessary but not sufficient to account for the activation mode of RasGRP3 in BCR signaling context.

In terms of an additional mechanism, because GEFs are known to be subjected to multiple levels of regulation, including phosphorylation both on serine/threonine, as in the case of Tiam1 (12), and on tyrosine, as in the case of Vav and Ras-GRF1 (13–15), one attractive possibility is that a protein kinase, downstream of PLC-γ2, regulates RasGRP3 through a phosphorylation mechanism. In fact, this possibility is suggested by previous experiments using pharmacological inhibitors; PKC inhibitors influenced RasGRP3 phosphorylation status as well as Ras–extracellular signal-regulated kinase activation in B cells, although a direct causal relationship between RasGRP3 phosphorylation and Ras activation was lacking (16). We report here that, in addition to recruitment, enzymatic activation of RasGRP3 through phosphorylation at Thr-133 is required for optimal Ras activation in BCR signaling.

Materials and Methods

Cells, Abs, and Reagents. Wild-type and mutant DT40 cells were maintained in RPMI medium 1640 (Invitrogen) supplemented with 10% FCS, 1% chicken serum, 50 μM 2-mercaptoethanol, 4 mM L-glutamate, and antibiotics. 293T cells were cultured in DMEM (Invitrogen) supplemented with 10% FCS and antibiotics. Establishment of RasGRP3-deficient DT40 cells was described in ref. 10. Stimulation of DT40 cells through BCR was carried out by using 5 μg/ml anti-chicken IgM mAb (M4) (17). Anti-phospho Thr-133 Ab was obtained by immunizing rabbits with a synthesized peptide, CWMRRV(p-T)QRKKI. Anti-chicken RasGRP3 Ab was described in ref. 10. Anti-pan Ras mAb was purchased from Oncogene Science. Anti-PKC-β Ab and anti-extracellular signal-regulated kinase Ab were purchased from Santa Cruz Biotechnology. An inhibitor for conventional PKC (Go6976) was purchased from Calbiochem. For evaluating surface expression of BCR on various mutant DT40 cells, cells were stained with FITC-conjugated anti-chicken IgM Ab (Bentyl) for 20 min on ice. After being washed with PBS, cells were analyzed by FACSCalibur (Becton Dickinson).

Expression Constructs and Transfection. Chicken RasGRP3 cDNAs harboring a single amino acid mutation (see Fig. 2*A*) were generated by using QuikChange (Stratagene) according to the protocols provided by the manufacturer. A RasGRP3 GEF mutant construct was already made (10). T133A RasGRP3 was fused at its COOH terminus to enhanced GFP (EGFP). Various mutant cDNAs were cloned into pApuro expression vector and transfected into wild-type or RasGRP3-deficient DT40 cells by electroporation (550 V, 25 μF). Drug-resistant clones were selected by adding 0.5 μg/ml puromycin to culture. Mouse PKC-β2 cDNA was obtained by RT-PCR and cloned into pApuro expression vector. Transient transfection using 293T cells was performed by using Fu-

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Abbreviations: BCR, B cell receptor; DAG, diacylglycerol; GEF, guanyl nucleotide exchange factor; PLC, phospholipase C; EGFP, enhanced GFP.

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GENE6 reagent (Roche Applied Science, Indianapolis) according to manufacturer protocols.

Immunoprecipitation and Western Blot Analysis. For immunoprecipitation, cells were solubilized in Nonidet P-40 lysis buffer supplemented with protease and phosphatase inhibitors as described in ref. 18. Lysates were incubated with Sepharose beads coupled with anti-RasGRP3 Abs. In some experiments, immunoprecipitates were incubated with calf intestine alkaline phosphatase (Takara Shuzo, Kyoto) before being subjected to SDS/PAGE. Immunoprecipitates or whole-cell lysates were resolved on SDS/PAGE, transferred to a polyvinyldifluoride membrane (Bio-Rad), and detected by indicated Abs. Ras-GTP assay was performed as described in ref. 19.

Visualization of Intracellular Distribution of RasGRP3-EGFP. Microscopic analysis for visualizing intracellular distribution of RasGRP3-EGFP was carried out as described in ref. 20. In brief, DT40 cells stably expressing either wild-type or T133A RasGRP3-EGFP were incubated with 5 μg/ml M4 at room temperature for an indicated period. Fluorescence images were acquired with a confocal laser scanning microscope (FV500, Olympus, Melville, NY) using the 488-nm line of an argon laser for excitation and a 505-nm long-pass filter for emission.

Searching Putative Phosphorylation Sites by Search Engine and Construction of Structure Model. The motif for phosphorylation by conventional PKCs, T/S-X-R/K in chicken RasGRP3 protein, was first searched by using GENETYX software (Genetyx, Tokyo). Then, the probability for undergoing phosphorylation was evaluated by NETPHOS 2.0 (www.cbs.dtu.dk/services/NetPhos). A three-dimensional structure of the cdc25 and Ras exchange motif (REM) domains of RasGRP3 was built by homology modeling based on a crystal structure of Sos (21) as a template. In the case of chicken RasGRP3, the REM and cdc25 domains correspond to the regions of amino acid residues 1–129 and 130–399, respectively. A sequence alignment between RasGRP3 and Sos was carried out by using the CLUSTALW program (22). Based on the sequence alignment, the model structure was constructed by using the MODELLER program (http://salilab.org/modeller) (23).

Results

RasGRP3 T133A Phosphorylation Mutant Is Defective in BCR-Mediated Ras Activation. The previous study showing that RasGRP3 undergoes phosphorylation upon BCR cross-linking in Ramos B cells (16) promoted us to examine whether this phosphorylation also occurs in DT40 B cells. As shown in Fig. 1, DT40 B cells exhibited a significant shift in the electrophoretic mobility of RasGRP3 upon BCR stimulation (second lane). This is presumably because of phosphorylation, because the stimulated-RasGRP3 protein was converted to a faster-migrating form by treatment with a phosphatase (sixth lane). Furthermore, in good accordance with the data reported in ref. 16, Go6976, which is a selective inhibitor for conventional PKC isozymes (24, 25), was effective at blocking the RasGRP3 mobility shift as well as Ras activation in the BCR-signaling context (fourth lane).

Based on the above data, we searched the chicken RasGRP3 sequence with the protein database motif engine NETPHOS 2.0 to identify its putative phosphorylation sites mediated by conventional PKC isozymes. Four potential residues, Thr-133, Ser-139, Ser-167, and Ser-601, gave a significant score. It should be noted that these four sites are conserved in human, mouse, and chicken, and that the more N-terminal three sites (Thr-133, Ser-139, and Ser-167) are located in the cdc25 domain of RasGRP3 (Fig. 2*A*) (11).

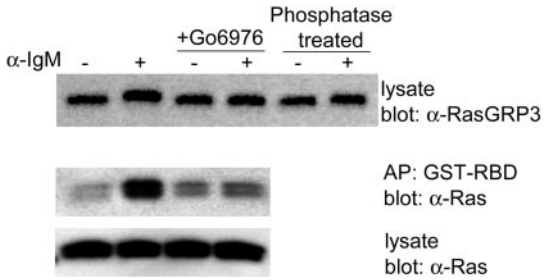

Fig. 1. Phosphorylation of RasGRP3 by BCR stimulation and its correlation with Ras activation. DT40 cells were incubated at 37°C with or without 20 μM Go6976 for 10 min, followed by stimulation with 5 μg/ml M4 for 3 min. Cell lysates were prepared and incubated with anti-chicken RasGRP3 Ab-coupled beads for 1 h. Beads were either left untreated or treated with 20 units of calf intestine alkaline phosphatase for 30 min at 37°C. Proteins bound to beads were eluted and subjected to Western blot analysis using anti-chicken RasGRP3 Ab. A total of 1×10^7 cells per lane was used (*Top*). For measuring active GTP-bound Ras, lysates (1×10^7 cells per lane) were incubated with RBD-bound beads for 30 min. Protein eluted from beads was subjected to Western blot analysis using anti-Ras mAb (*Middle*). As demonstrated in ref. 10, the upper and lower bands of Ras are likely to represent K-Ras and H-Ras, respectively. The lysates (1×10^6 cells per lane) also were subjected directly to Western blot analysis for measuring total amount of Ras protein (*Bottom*).

To test whether these potential phosphorylation modifications alter RasGRP3 function, each site was mutated, and the resulting mutants were expressed in RasGRP3-deficient DT40 B cells. All mutant proteins were stable, indicating that these mutations do not significantly interfere with RasGRP3 folding and/or degradation (Fig. 2*C*). Among these mutants, only the T133A mutation caused a major defect in BCR-mediated Ras activation; this decrease cannot be ascribed to decreased expression of BCR (Fig. 2 *B* and *D*). Therefore, we focused our study on the regulation of RasGRP3 function through Thr-133 phosphorylation.

Before BCR stimulation (0 min in Fig. 2*D*), slightly increased Ras activation of the S139A and S601A mutants was reproducibly observed, suggesting that these sites might undergo phosphorylation before receptor stimulation, thereby negatively controlling Ras activation.

Thr-133 Is Phosphorylated After BCR Stimulation. To verify Thr-133 phosphorylation of RasGRP3, we first prepared anti-pT133 RasGRP3 Abs and examined specificity by using 293T cells. Because the above data suggest the involvement of conventional PKCs in RasGRP3 phosphorylation, wild-type RasGRP3 or its T133A mutant was cotransfected with PKC-β into 293T cells, and the cell lysates were immunoprecipitated and blotted with anti-pT133 or anti-RasGRP3 Ab. As demonstrated in Fig. 3*A*, although equal amounts of RasGRP3 protein were precipitated from both samples, anti-pT133 Ab reacted with wild-type RasGRP3 (second lane) but not its mutant (fourth lane). Moreover, this reactivity of wild-type RasGRP3 was completely abolished by phosphatase treatment (third lane). Even in the absence of exogenous PKC-β, a certain level of phosphorylation at Thr-133 was observed (first lane), implying that endogenous conventional PKCs in 293T cells are capable of phosphorylating Thr-133.

The data obtained by 293T cells were further substantiated by experiments using RasGRP3-deficient DT40 transfectants expressing wild-type RasGRP3 or its T133A mutant; wild-type RasGRP3, but not its mutant, was recognized by anti-pT133 Ab in a BCR-dependent manner (Fig. 3*A*).

To investigate whether this site is indeed phosphorylated in endogenous RasGRP3, DT40 B cells were stimulated with BCR

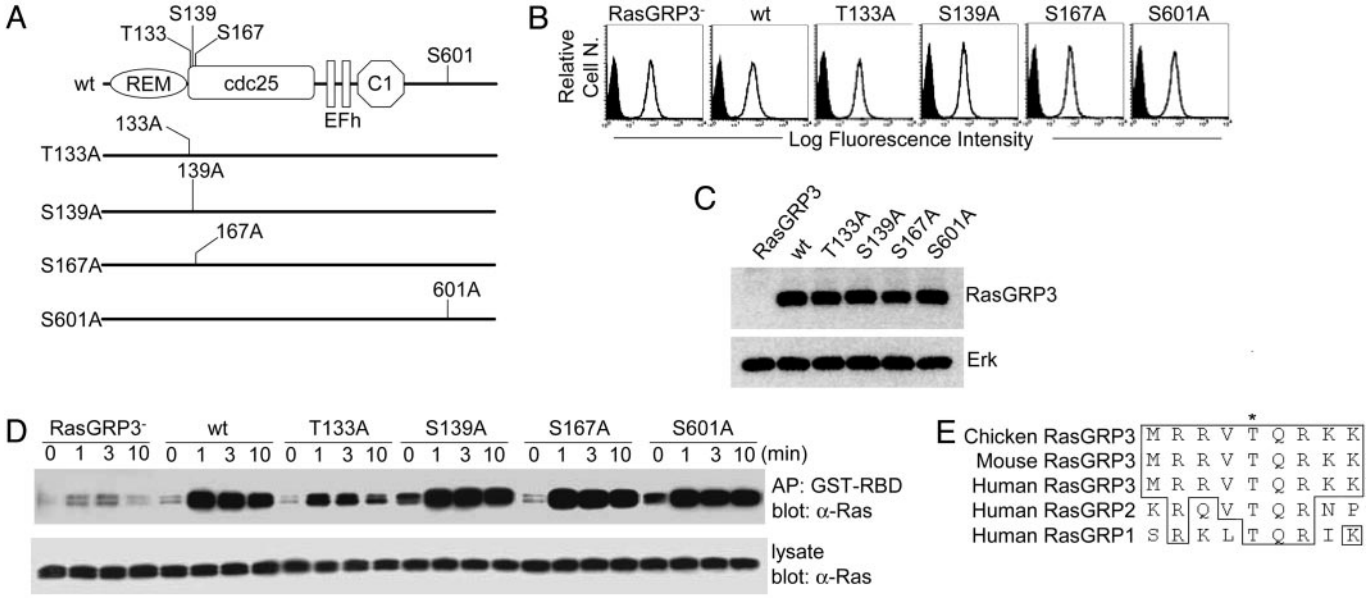

Fig. 2. Thr-133 in RasGRP3 is crucial for BCR-mediated Ras activation. (*A*) Schematic diagram of RasGRP3 mutants. By homology modeling based on structures of Sos, an N-terminal ≈400-residue catalytic module of chicken RasGRP3 is thought to be sufficient for the Ras-specific nucleotide exchange activity. This segment of RasGRP3 includes a core region known as the cdc25 domain (residues 130–399) and an additional domain, known as the Ras exchange motif domain, located N-terminal to the cdc25 domain (residues 1–129). RasGRP3 cDNAs encoding these mutations were transfected into RasGRP3-deficient DT40 cells. A single clone expressing each RasGRP3 mutant was extensively analyzed and represented, although some critical experiments were carried out, at least, using two different clones. EFh, EF hand; C1, protein kinase C conserved region 1 domain. (*B*) Expression of surface BCR on various mutant DT40 cells. Filled and opened histograms represent unstained cells and cells stained with FITC-anti-chicken IgM, respectively. (*C*) Expression of RasGRP3 in various mutant DT40 cells. Whole-cell lysates (2×10^6 cells per lane) were subjected to Western blot analysis using anti-chicken RasGRP3 Ab (*Upper*) or anti-extracellular signal-regulated kinase Ab (*Lower*). (*D*) BCR-mediated Ras activation in various mutant DT40 cells. DT40 B cells (1×10^7 cells per lane) were stimulated with M4 (5 μg/ml) for the indicated period. Lysates were incubated with RBD-bound beads for measuring GTP-bound Ras. Proteins eluted from beads were resolved in SDS/PAGE followed by Western blot analysis using anti-Ras mAb (*Upper*). For measuring total Ras, parts of the lysates (1×10^6 cells per lane) were subjected to Western blot analysis without incubation with beads (*Lower*). (*E*) Comparison of protein sequences of the RasGRP family surrounding Thr-133 (denoted by an asterisk).

for different time points, after which endogenous RasGRP3 was immunoprecipitated and blotted with anti-pT133 Ab. As shown in Fig. 3*B*, endogenous RasGRP3 was phosphorylated at Thr-133 in a BCR-dependent manner. Deletion of PLC-γ2 or treatment of Go6976 significantly inhibited phosphorylation at Thr-133 in DT40 B cells, suggesting that this phosphorylation is mediated by conventional PKC isoforms in a BCR-signaling context.

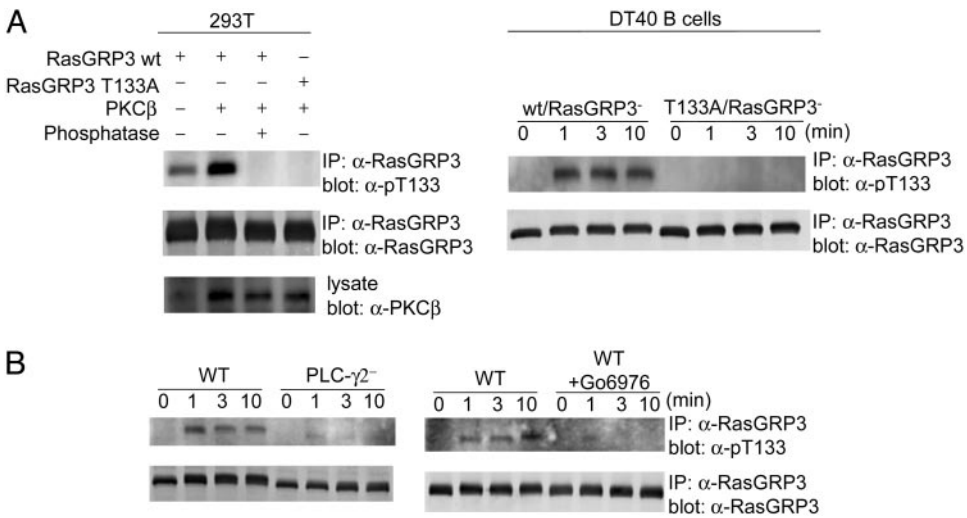

Fig. 3. Phosphorylation of RasGRP3 Thr-133. (*A*) Wild-type RasGRP3 or its T133A mutant was coexpressed with PKC-β in 293T cells, and immunoprecipitated RasGRP3 was blotted with anti-pT133 Ab (*Left*). RasGRP3 or its mutant was immunoprecipitated from RasGRP3-deficient DT40 B cells expressing exogenous wild-type RasGRP3 or its T133A mutant (Fig. 2*C*) and blotted with anti-pT133 Ab (*Right*). (*B*) Wild-type or PLC-γ^2-deficient DT40 B cells were stimulated, immunoprecipitated, and blotted with anti-pT133 Ab (*Left*). Wild-type DT40 cells were treated with Go6976 for 10 min or left untreated, then stimulated by means of BCR (*Right*).

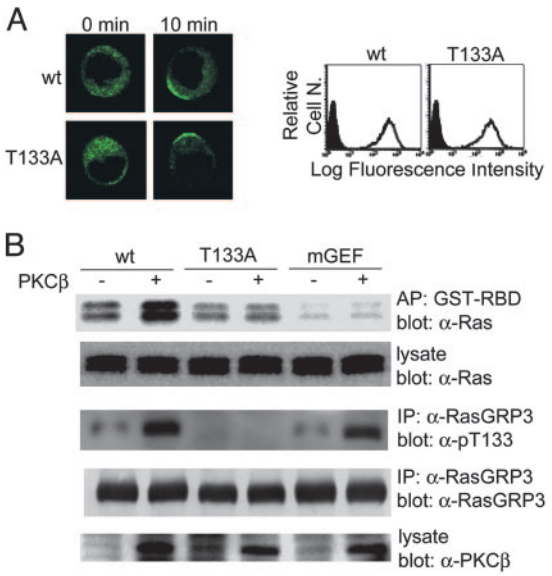

Fig. 4. Effect of RasGRP3 T133A mutation on its activation. (*A*) DT40 cells expressing wild-type RasGRP3-EGFP or its T133A mutant were stimulated by means of BCR, and subcellular localization of fusion proteins was visualized by using a confocal microscope. Among 50 cells counted for membrane localization, the numbers that showed clear membrane localization were 37 for DT40 cells expressing wild-type RasGRP3-EGFP and 32 for those expressing T133A mutant (*Left*). The amount of fusion proteins expressed in each transfectants was evaluated by flow cytometric analysis (*Right*). (*B*) Wild-type RasGRP3 or its mutants were coexpressed with PKC-β in 293T cells, and the Ras activation status was measured by using RBD-bound beads.

Phosphorylation of T133 Is Required for Enzymatic Activation of RasGRP3. As demonstrated in Fig. 2 *C* and *D*, despite the similar expression level between RasGRP3 T133A mutant and its wild type, T133A mutant exhibited a defect in BCR-mediated Ras activation. However, when compared with RasGRP3-deficient parental DT40 cells, this mutant displayed a significant increase in Ras activation, suggesting that another phosphorylation site might participate in RasGRP3 activation in combination with Thr-133 and/or that a phosphorylation-independent mechanism contributes to this residual activation in the case of the T133A mutant.

Ras activation is thought to require recruitment of RasGRP3 to the membrane, where it interacts with Ras (10, 26, 27). Thus, we examined the possibility that phosphorylation at Thr-133 of RasGRP3 might directly or indirectly affect its recruitment upon BCR stimulation. As demonstrated in Fig. 4*A*, like wild-type RasGRP3-EGFP, its mutant EGFP began to move to the membrane after 1 min of BCR stimulation, and this membrane association persisted until at least 10 min after stimulation, demonstrating nonnecessity of Thr-133 phosphorylation in RasGRP3 recruitment. Hence, we examined the second possibility that phosphorylation at Thr-133 of RasGRP3 might affect its enzymatic activity. For this purpose, wild-type RasGRP3 or its T133A mutant was introduced into 293T cells. When PKC-β was cotrasfected with RasGRP3, as described above, wild-type RasGRP3, but not the T133A mutant, underwent a significant increase in phosphorylation at Thr-133, as demonstrated by using anti-pT133 Ab. As revealed in Fig. 4*B*, the extent of this phosphorylation at Thr-133 was well correlated with that of active Ras in these conditions, suggesting a connection between Thr-133 phosphorylation and its GEF activity. Taken together, these findings suggest that Thr-133 in RasGRP3, after being phosphorylated by presumably conventional PKCs, contributes to its enzymatic activation rather than recruitment to the membrane.

Discussion

In this study, we identify a Thr phosphorylation site (Thr-133) in the cdc25 domain of RasGRP3 that is essential for BCR-mediated Ras activation. Given the importance of membrane recruitment of RasGRP3 in its activation, as shown by our previous study as well as other laboratories (10, 26, 27), these data strongly suggest that maximal activation of RasGRP3 involves the cooperative action of at least two signals: one recruiting the RasGRP3 to the plasma membrane and the other phosphorylating it.

In addition to the plasma membrane localization of RasGRP3, recent reports have indicated the possibility that RasGRP3 also moves to the Golgi complex, where it activates Ras (28, 29). However, phosphorylation at Thr-133 and the subsequent activation of RasGRP3 probably take place at the plasma membrane, rather than the Golgi complex, because of the following observations. In DT40 B cells, after initial movement of RasGRP3 to the plasma membrane, then it localized at the perinuclear region, presumably Golgi complex in a relatively late phase (after $\approx$15 min of BCR stimulation) (data not shown). Accompanying the early movement to the plasma membrane, RasGRP3 underwent phosphorylation at Thr-133, as observed after 1 min of BCR stimulation (Fig. 3). Moreover, the defect by T133A mutation was manifested in the early phase (Fig. 2).

Although PKC-δ is suggested to participate in RasGRP3 phosphorylation (30), four lines of evidence favor the idea that the kinase mainly responsible for Thr-133 phosphorylation is most likely PKC-β in B cells: (*i*) direct phosphorylation of RasGRP3 by PKC-β *in vitro* (16); (*ii*) Thr-133 phosphorylation of RasGRP3 by its coexpression with PKC-β in 293T cells (Fig. 3*A*); (*iii*) reduction of Thr-133 phosphorylation by treatment of Go6976, an inhibitor for conventional PKC isozymes, in BCR stimulated-B cells (Fig. 3*B*); and (*iv*) apparently normal Ras activation in PKC-δ-deficient DT40 B cells (Y.A. and T.K., unpublished data).

PKC-β, like RasGRP3, possesses a C1 domain whose interaction with DAG is responsible for membrane recruitment. Assuming that PKC-β is a kinase responsible for Thr-133 phosphorylation, the data presented here support the proposed model that DAG generated upon BCR engagement facilitates recruitment of both PKC-β and RasGRP3 to the plasma membrane, wherein PKC-β phosphorylates Thr-133 in RasGRP3, being crucial for full activation of RasGRP3 (16). Because RasGRP1, dominantly expressed in T cells (27), has homologous Thr in its cdc25 domain (Fig. 2*E*), we propose that similar mechanisms also operate in the case of TCR.

The T133A mutant partially restored BCR-mediated Ras activation, when compared with RasGRP3$^{-/-}$ parental DT40 cells, which could be explained by the following possibilities. First, there appear to exist multiple phosphorylation sites on RasGRP3, because the RasGRP3 T133A mutant still exhibited an electrophoretic mobility shift upon BCR stimulation in DT40 B cells (Fig. 3*A*). A certain level of RasGRP3 activation upon BCR engagement might occur by phosphorylation at other sites, which could be mediated by nonconventional PKC(s). In this regard, our results cannot exclude the possibility that full activation of RasGRP3 requires nonconventional PKCs, in addition to conventional PKCs. Second, as proposed in the case of PKC-γ, binding of DAG to the C1 domain of RasGRP3 could serve two purposes: (*i*) increasing the binding affinity of RasGRP3 with the plasma membrane and (*ii*) enhancing the enzymatic activity to some extent (31). According to this idea, the partial restoration by the T133A RasGRP3 mutant might be provided by modest activation through its intact C1 domain. Additional studies are

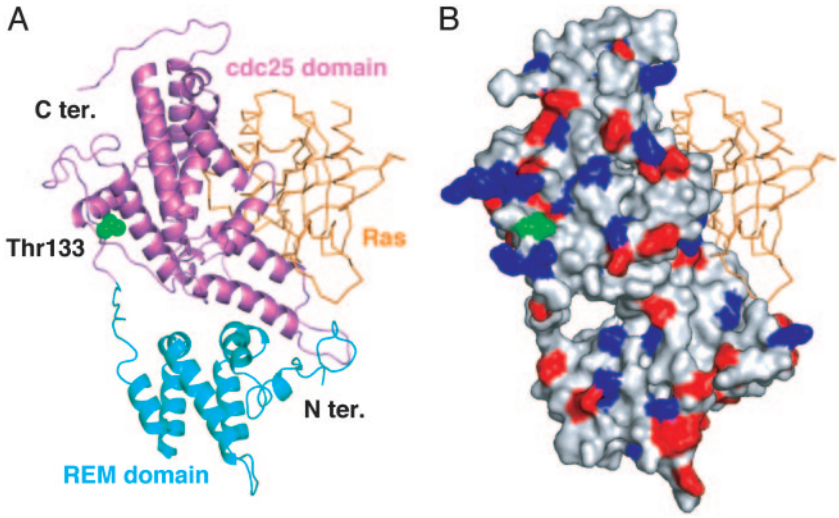

Fig. 5. Model structure of RasGRP3. The RasGRP3 structure was built by homology modeling based on a crystal structure of Sos. (*A*) Ribbon models of Ras exchange motif (cyan) and cdc25 (pink) domains of chicken RasGRP3. Ras (orange) is put on an equivalent position by superimposition of the model on Sos structures. Sphere (green) indicates the phosphorylation site at Thr-133. (*B*) Molecular surface of RasGRP3 is shown. Red and blue indicate negatively charged residues, Glu and Asp, and positively charged residues, Lys and Arg, respectively. The orientation is same as in *A*.

under way to define which possibility is more likely in DT40 B cells.

A three-dimensional model structure of the Ras-GEF domain of RasGRP3 was built by homology modeling based on a crystal structure of Sos (Fig. 5) (21). According to this model, Thr-133 is located in a flexible loop opposite to the Ras binding site in the cdc25 domain, presumably corresponding to the active site of RasGRP3, and the surrounding region at Thr-133 consists of a cluster of positively charged amino acid residues (Fig. 5*B,* blue). Thus, introducing a negative charge through phosphorylation at Thr-133 could have a big impact of the electrostatic potential around the flexible loop, possibly causing a key change in the conformation of RasGRP3. Based on the above consideration, several potential mechanisms by which Thr-133 phosphorylation alters RasGRP3 function are envisaged. First, this conformational change could involve reorientation of the cdc25 domain and stabilization of the active site, thereby enhancing the GDP/GTP exchange reaction. Indeed, this type of allosteric regulation by phosphorylation distal to the active site was reported in the case of glycogen phosphorylase (32). Second, given the evidence that many GEFs including Sos contain a regulatory domain that blocks activity through an intramolecular interaction (33), the surrounding region around Thr-133, might form such an interaction with other domains of RasGRP3. Hence, disruption of the interaction by phosphorylation at Thr-133 may relieve this autoinhibition, thereby promoting enzymatic activation. Finally, phosphorylation at Thr-133 in the cdc25 domain might regulate RasGRP3 activity by modulating binding to cellular regulatory molecules. In these contexts, the identification of sequences that contribute to the repression of RasGRP3 activity, or of B cell proteins that interact with the surrounding region around Thr-133, should further the understanding regulatory mechanisms of RasGRP3 within the Ras pathway.

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