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Possible Involvement of Shc in IL-4-Induced Germline ϵ Transcription in a Human B Cell Line

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The IL-4Rα contains the I4R motif which binds to the phosphotyrosine binding domain of several adaptor proteins, including IRS-1/2 and Shc. Although the involvement of IRS-1/2 in IL-4-induced PI3-kinase activation is known, there is little information on the role of Shc in IL-4 signaling. In this study, we found the preferential utilization of Shc by the IL-4Rα in a human Burkitt's B lymphoma cell line, DND39. IL-4 induced the association of tyrosine-phosphorylated Shc with the IL-4Rα, whereas no detectable tyrosine phosphorylation of IRS-1 or IRS-2 was induced. IL-4-induced germline ϵ promoter activation was enhanced by overexpression of Shc and was inhibited by truncated Shc lacking the collagen-homologous domain. We further found the association of Shc with PLCγ1. Although direct tyrosine phosphorylation of PLCγ1 was not detectable, the amount of PLCγ1 coprecipitable with anti-phosphotyrosine was increased after IL-4 stimulation. These results suggest that Shc can function as an adaptor protein of the IL-4Rα and mediate the germline ϵ transcription.

Interleukin-4 (IL-4) is a pleiotropic cytokine that mediates a diverse array of functions in cells of hematopoietic as well as non-hematopoietic origin (1). The multifunctional role of IL-4 is reflected by the ubiquitous expression of the IL-4 receptor (IL-4R) (2). Whereas the IL-4R α chain (IL-4Rα) alone can form a tight complex with its ligand, functional IL-4R is composed of the IL-4Rα and either the common γ chain (γc) or the IL-13Rα, all of which lack the intrinsic tyrosine kinase domain (3–7). However, IL-4 stimulation results in increased tyrosine phosphorylation of several cellular proteins, including the IL-4Rα (8). Many cytokines activate members of the Jak family tyrosine kinases, which subsequently tyrosine-phosphorylated transcriptional factors called Stats. IL-4 activates Jak1 and Jak3 and induces activation of Stat6 (9, 10). Recent studies utilizing Stat6 knockout mice have established that Stat6 is required for various functional responses induced by IL-4 (11).

In addition to the Jak-Stat pathway, IL-4 also induces tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and/or IRS-2 (8, 12–14), which mediate the activation of phosphatidylinositol 3-kinase (PI3-kinase). The importance of this signaling pathway in the proliferative response to IL-4 has been demonstrated in 32D cells (13. The phosphorylation of IRS-1/2 has also been observed in many cell types, including primary human T cells as well as murine T and B cells (15, 16). However, IRS-1/2 is not tyrosine-phosphorylated in several IL-4-responsive cells; neither IRS-1 nor IRS-2 is expressed at detectable levels in murine primary bone marrow mast cells or in the IL-4-dependent T cell line, CT.4S, and IL-4 fails to induce tyrosine phosphorylation of IRS-1 or IRS-2 in the murine T cell lines, CTLL-2 and HT2, as well as in Epstein–Barr virus-immortalized human B cells (16–18). These findings suggest that another molecule besides IRS-1/2 is involved in IL-4 signal transduction.

IRS-1 and IRS-2 bind to the insulin/IL-4R (I4R) region of the IL-4Rα. This region contains NPXY motif which is also present in the insulin receptor and interacts with phosphotyrosine binding (PTB) domain of IRS-1/2 (14, 19, 20). Like IRS-1/2, Shc contains the PTB domain, and recombinant Shc can bind to a phosphopeptide identical to the I4R region, despite the negligible sequence homology between the PTB domain of IRS-1/2 and that of Shc (21, 22). The ability of Shc to bind to the I4R motif was also confirmed in the yeast two-hybrid system (23). However, the functional significance of Shc in IL-4 signaling was not clearly shown in intact cells.

The present study demonstrates that in a human Burkitt's B lymphoma cell line, DND39, IL-4 induced the association of tyrosine-phosphorylated Shc with the IL-4Rα, while neither IRS-1 nor IRS-2 was

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tyrosine-phosphorylated by IL-4. We further found the constitutive association of Shc with phospholipase Cγ1 (PLCγ1). Our results suggest that the IL-4Rα preferentially binds to Shc, rather than IRS-1/2, depending on the cell type, and Shc functions as a docking protein in IL-4 signal transduction.

MATERIALS AND METHODS

Cells and reagents. The human Burkitt's lymphoma B cell line DND39 was kindly provided by Fujisaki Cell Center (Hayashibara Biochemical Laboratories Inc., Okayama, Japan) and was maintained in RPMI1640 medium supplemented with 10% FCS. HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% FCS. Recombinant human IL-4 and the anti-human IL-4Rα monoclonal antibody were purchased from Genzyme (Cambridge, MA). Anti-Shc monoclonal antibody and polyclonal anti-Shc and anti-PLCγ1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-IRS-1 and anti-IRS-2 antibodies, as well as anti-phosphotyrosine (4G10) and biotinylated 4G10 monoclonal antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). $F(ab')_2$ fragment of goat anti-human IgM antibody was purchased from from Tago (Burlingame, CA). Recombinant GST and GST fusion protein containing the SH2–SH2–SH3 region of rat PLCγ1 (amino acids 530–850) were purchased from Santa Cruz. The amino acid homology of this region is 98% between man and rat.

Immunoprecipitation and immunoblotting. The cells were lysed with lysis buffer containing 1% Nonidet P-40, 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The lysates were precleared with protein G–Sepharose (Pharmacia, Uppsala, Sweden) at 4°C for 1 h and incubated with the appropriate antibody at 4°C for 4 h. Immunocomplexes were precipitated with protein G-sepharose at 4°C for 1 h, washed with lysis buffer, eluted by boiling in SDS–PAGE sample buffer containing 2-ME, resolved on SDS–PAGE, and transferred to PVDF membranes (Bio-Rad, Hercules, CA). Proteins were probed with appropriate antibodies and visualized by enhanced chemiluminescence (ECL, Amersham).

Plasmids. A fragment of the human germline ϵ promoter from position −214 to +36 (24) was amplified using primers TAGACTGGATCCAGCACTCGGTGTGCATCG (sense) and TAGACTAAGCTTGTCCGGCTCCCTGTCGGG (antisense). The amplified products were digested with *Bam*HI and *Hin*dIII, and inserted into the promoterless firely luciferase vector, pGL3-Basic (Promega, Madison, WI), digested with *Bgl*II and *Hin*dIII. To generate Shc expression vector, cDNA encoding a 46-kDa isoform of Shc (25) was obtained by standard RT-PCR amplification and cloned into pTARGET expression vector (Promega). The deletion of the PTB domain (aa 7–165), the collagen-homologous (CH) domain (aa 188–332), and the SH2 domain (aa 333–422) of Shc were accomplished using the GeneEditor *in vitro* site-directed mutagenesis system (Promega).

Transfection. DND39 cells (2×10^6) were suspended in Iscov's modified Dulbecco's medium (GIBCO BRL, Grand Island, NY) containing 5% FCS and seeded per well of a 6-well plate. The cells were transiently transfected with 1 μg of germline ϵ reporter plasmid plus 0.4 μg of either Shc expression or an empty vector using Effectene transfection reagent (QIAGEN, Germany) according to the manufacturer's instruction (DNA:Effectene ratio of 1:10). To control for transfection efficiencies, 40 ng of pRL-SV40 (Promega) encoding sea pansy luciferase was contransfected. After 24 h of transfection, the cells were further incubated with or without IL-4 for 24 h and harvested for luciferase assay using dual-luciferase reporter assay system (Promega).

Mass analysis of IP_3. Cells (1×10^6 cells/ml) were stimulated either with IL-4 (250 U/ml) or with $F(ab')_2$ fragment of anti-human IgM antibody (10 μg/ml) for the indicated times and reactions were stopped by addition of 20% perchloric acid (0.2 volume). After 20 min on ice, cell debris was removed by centrifugation, and the supernatants were neutralized by addition of a mixture of tri-*n*-octylamine and 1,1,2-trichlorofluoroethane (1:1, v/v). After centrifugation, the upper phases were subjected to IP_3 analysis using a [^{3}H] IP_3 assay kit (Amersham).

GST fusion protein binding assay. The cells were lysed with lysis buffer containing 1% digitonin, 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The lysates were precleared with glutathione-Sepharose (Pharmacia) at 4°C for 1 h and incubated either with GST fusion protein containing the SH2–SH2–SH3 region of bovine PLCγ1 or with GST alone at 4°C for 4 h. The complexes were precipitated with glutathione–Sepharose at 4°C for 2 h, washed with lysis buffer, eluted by boiling in SDS–PAGE sample buffer containing 2-ME, resolved on SDS–PAGE, transferred to PVDF membranes (Bio-Rad), and immunoblotted as described above.

RESULTS

IL-4 does not induce the tyrosine phosphorylation of IRS-1 and IRS-2 in DND39 cells. We have previously shown that induction of germline ϵ transcription in DND39 cells by IL-4 requires the activation of PI3-kinase (26). However, anti-phosphotyrosine blot of the lysates from the stimulated cells did not show the band corresponding to IRS-1 and IRS-2. To determine whether this is due to the failure of tyrosine phosphorylation of IRS-1 and IRS-2 or to the absence of these proteins, the lysates from the IL-4-stimulated cells were immunoprecipitated with respective antibodies and blotted with anti-phosphotyrosine antibody. As shown in Fig. 1, neither IRS-1 nor IRS-2 was tyrosine-phosphorylated after IL-4 stimulation, whereas both proteins were detected by reprobing the same blot with anti-IRS-1 and anti-IRS-2 antibodies, respectively. The failure to detect tyrosine phosphorylation of IRS-1/2 was not due to the technical problems, because tyrosine phosphorylation of IRS-1 was detected in IL-4-stimulated HeLa cells.

Association of Shc with the IL-4Rα in DND39 cells. Because Shc is a candidate which binds to the I4R motif of the IL-4Rα, we examined whether Shc is associated with the IL-4Rα following IL-4 stimulation. Anti-IL-4Rα immunoprecipitates were prepared from the cells and sequentially probed with anti-phosphotyrosine and anti-Shc antibodies. An enhanced tyrosine phosphorylation of an approximately 50-kDa protein was detected in the IL-4-stimulated cells, compared to the unstimulated cells (Fig. 2A). The same band was also recognized by anti-Shc antibody (Fig. 2B), which corresponded to a 46-kDa isoform of Shc ($p46^{Shc}$). In these experiments, we were unable to detect the tyrosine-phosphorylation of IL-4Rα (approximately 140 kDa). The difficulty in detecting tyrosine-phosphorylated IL-4Rα was described previously and suggested to be due to its susceptibility to phosphatase activity (27). These results indicated that the IL-4Rα

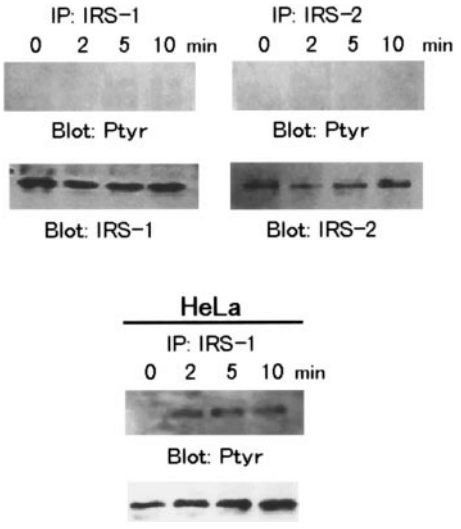

FIG. 1. Failure of IL-4 to induce tyrosine phosphorylation of IRS-1 and IRS-2 in DND39 cells. DND39 cells (2×10^7 cells) and HeLa cells were stimulated with or without IL-4 (250 U/ml) for the indicated times and lysed with 1% NP-40. The lysates were immunoprecipitated with anti-IRS-1 or anti-IRS-2 antibody and subjected to immunoblot analysis with biotinylated anti-phosphotyrosine antibody plus peroxidase-conjugated avidin. Expression of IRS-1 and IRS-2 was examined by reprobing the same membrane with anti-IRS-1 and anti-IRS-2 antibodies, respectively.

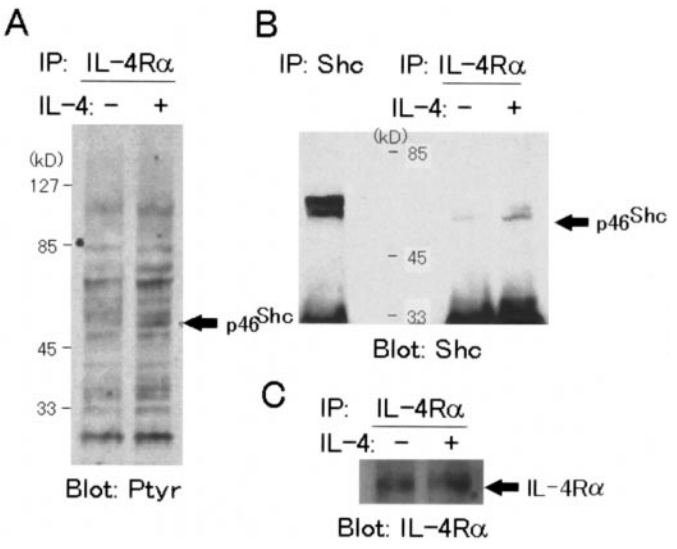

FIG. 2. Tyrosine-phosphorylated Shc is associated with IL-4Rα in DND39 cells. The cells (3×10^7 cells) were stimulated with IL-4 (250 U/ml) for 5 min and lysed with 1% NP-40. The lysates were immunoprecipitated with anti-IL-4Rα antibody and blotted with biotinylated anti-phosphotyrosine antibody (A). After stripping, appropriate membrane fractions were cut and blotted with either (B) polyclonal anti-Shc antibody or (C) anti-IL-4Rα antibody to confirm equal amounts of precipitation. Anti-Shc immunoprecipitates run in parallel are shown in B to denote the position of $p46^{Shc}$.

preferentially binds to Shc, rather than IRS-1 and IRS-2, in IL-4-stimulated DND39 cells.

Role of Shc in IL-4-induced germline ϵ transcription in DND39 cells. It is known that DND39 cells express germline ϵ mRNA in response to IL-4 (24). To analyze the involvement of Shc in IL-4 signaling, DND39 cells were transiently transfected with germline ϵ reporter plasmid together with an empty plasmid or Shc expression plasmid. Since Shc is composed of the PTB, CH, and SH2 domains, we also transfected the Shc mutants lacking each domain. Figure 3 shows that IL-4 induced a 4.1-fold increase in germline ϵ promoter activity in mock-transfected cells. Overexpression of wild-type Shc gave rise to elevated IL-4-inducibility (6.2-fold), and deletion of the PTB domain abolished such an effect. The SH2 domain-deleted Shc enhanced the IL-4-inducibility as efficiently as wild-type Shc. Conversely, deletion of the CH domain diminished the germline ϵ promoter activity below the level of mock-transfected cells, suggesting that it behaves as a dominant negative mutant. The transcription level of luciferase reporter construct lacking germline ϵ promoter was not affected by the overexpression of Shc (data not shown).

Association of Shc with PLCγ1 in DND39 cells. It has been proposed that tyrosine-phosphorylated Shc forms a complex with Grb2, which appears to be responsible for the activation of ERK (28). However, IL-4 failed to activate ERK in DND39 cells (data not shown), in agreement with the previous reports using other cell types (28, 29). Then, we examined whether Shc is involved in the activation of PLC, because IL-4 induces inositol 1,4,5-triphosphate (IP_3) production in human B cells (30). When DND39 cells were stimulated with IL-4, the prolonged accumulation of IP_3 was observed over 10 min. The IP_3 levels in IL-4-stimulated

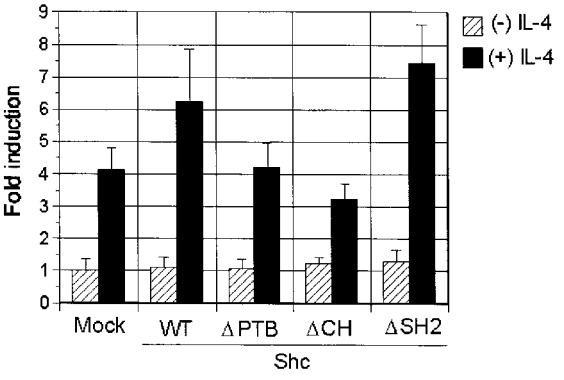

FIG. 3. Effect of expression of wild-type and truncated Shc in IL-4-induced germline ϵ transcription activity. DND39 cells were transiently transfected with germline ϵ reporter plasmid plus either an empty plasmid or expression plasmids for wild-type or truncated Shc. After 24 h, the cells were unstimulated (hatched bars) or stimulated with 250 U/ml of IL-4 (solid bars) for an additional 24 h and harvested for luciferase assay. Values are expressed as fold induction over the basal activity of unstimulated cells cotransfected with an empty expression vector. Results are mean values and SDs of three independent experiments.

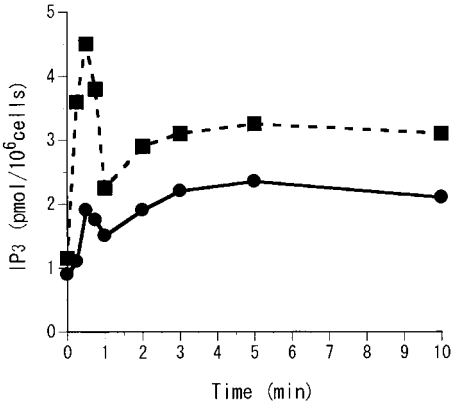

FIG. 4. IL-4 induces the production of IP_3 in DND39 cells. Cells were stimulated with 250 U/ml of IL-4 (circles) or 10 μg/ml of anti-IgM (squares) for the indicated times. IP_3 were extracted and measured as described under Materials and Methods. Results are mean values of three independent experiments.

cells were comparable to those observed in anti-IgM stimulated cells, although the initial IP_3 production (within 1 min) was pronounced in the anti-IgM stimulated cells (Fig. 4). Then, anti-PLCγ1 immunoprecipitates from IL-4-stimulated DND39 cells were probed with anti-phosphotyrosine antibody. Increased tyrosine-phosphorylation of a 50-kDa protein, which peaked at 5 min, was detected in the stimulated cells, although no increased tyrosine phosphorylation of PLCγ1 was detected (Fig. 5A). In addition, when anti-phosphotyrosine immunoprecipitates were blotted with anti-PLCγ1 antibody, an increase in the amount of PLCγ1 was observed in the stimulated cells (Fig. 5B), suggesting that IL-4 could induce changes in the intracellular distribution of PLCγ1. To characterize the 50-kDa protein in Fig. 4A, the anti-PLCγ1 immunoprecipitates were reprobed with anti-Shc antibody. As shown in Fig. 5C, the anti-Shc antibody recognized a band identical to the 50-kDa tyrosine-phosphorylated protein illustrated in Fig. 5A, although the same band was detected even in the unstimulated cells and its intensity did not change after IL-4 stimulation. To further confirm the ability of PLCγ1 to associate with Shc even in the absence of IL-4, GST fusion protein containing the SH2–SH2–SH3 region of PLCγ1 was used. As shown in Fig. 5D, this fusion protein could precipitate Shc from the lysates of both the unstimulated and the IL-4-stimulated cells. The levels of Shc association with the fusion protein was unaffected by IL-4 stimulation.

DISCUSSION

The present study demonstrates that IL-4 induces the association of tyrosine-phosphorylated Shc with the IL-4Rα in a human B cell line, DND39, whereas neither IRS-1 nor IRS-2 is tyrosine-phosphorylated

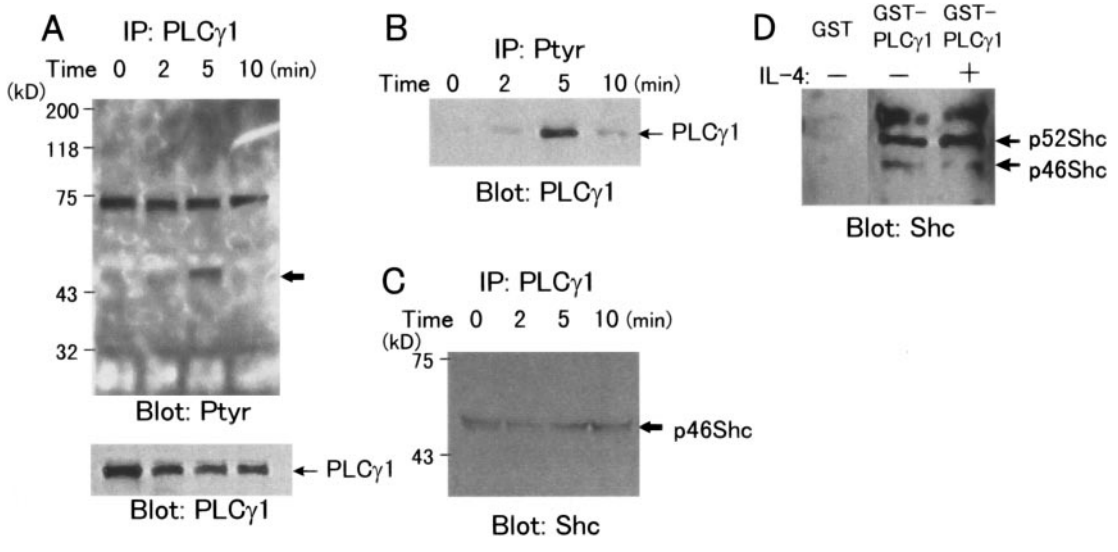

FIG. 5. Association of Shc with PLCγ1 in DND39 cells. (A) Lysates from DND39 cells (3×10^7 cells) stimulated with IL-4 (250 U/ml) for the indicated times were immunoprecipitated with anti-PLCγ1 antibody and subjected to immunoblot analysis with biotinylated anti-phosphotyrosine antibody plus peroxidase-conjugated avidin (upper panel). The signal around 70 kDa is nonspecific, because this band was not detected using unlabeled anti-phosphotyrosine antibody plus peroxidase-conjugated anti-mouse IgG. Equal amounts of precipitation were confirmed by reprobing the same membrane with anti-PLCγ1 antibody (lower panel). (B) The cells (8×10^7 cells) were stimulated with IL-4 (250 U/ml) for the indicated times and lysed with 1% NP-40. The lysates were immunoprecipitated with anti-phosphotyrosine monoclonal antibody and blotted with anti-PLCγ1 antibody. (C) The same membranes as in panel A were reprobed with monoclonal anti-Shc antibody. (D) DND39 cells (3×10^7 cells) were unstimulated or stimulated with IL-4 for 5 min. The lysates were incubated either with GST (10 μg) or with GST fusion protein containing SH2–SH2–SH3 region of PLCγ1 (10 μg). The complexes were precipitated with glutathione–Sepharose, resolved by SDS–PAGE, and then immunoblotted with anti-Shc antibody.

upon IL-4 stimulation. These results suggest that the IL-4Rα preferentially utilizes Shc rather than IRS-1/2, depending on the cell type. The ability of IL-4 induce tyrosine phosphorylation of Shc has been found in murine splenic B cells as well as in human keratinocytes (31, 32). However, it was not shown in these two cells whether tyrosine-phosphorylated Shc is associated with the IL-4Rα. Concerning the selectivity for Shc vs IRS-1/2, phosphopeptide binding assay showed that PTB domain of IRS-1 binds with higher affinity to the I4R motif of the IL-4Rα than that of Shc (21). It is therefore likely that there is a third component which facilitates the interaction between the IL-4Rα and Shc and that phosphotyrosine residues of Shc are necessary for this interaction. Identification and characterization of Shc-associated protein will clarify this possibility. The selectivity for Shc vs IRS-1/2 may also depend on the distinct reactivity of these molecules to tyrosine kinases. As Jak2 mediates tyrosine-phosphorylation of Shc in IL-3/GM-CSF signaling (33), some types of Jak kinase might be responsible for the phosphorylation of Shc.

Expression of germline ϵ mRNA is an essential step in the IgE class switching induced by IL-4. Thus, the involvement of Shc in IL-4 signaling was confirmed using a reporter construct driven by the germline ϵ promoter. Overexpression of Shc resulted in an elevation of IL-4-induced germline ϵ transcription activity, whereas expression of the CH domain-deleted Shc inhibited it, indicating that Shc is involved in this response and that its CH domain is important for downstream signaling. Although the binding sites for STAT6, C/EBP family, NF-κB and BSAP have been identified in the germline ϵ promoter (34), it is difficult to explore the connection between Shc and these transcriptional factors, because cooperation of multiple transcriptional factors is required for IL-4 induction, and tandem copies of individual elements alone cannot confer IL-4 responsiveness to a heterologous promoter (34–37). In keeping with this fact, reporter constructs, which contain two copies of STAT6 or NF-κB sites fused to a minimal promoter of herpes simplex virus thymidine kinase gene, were not activated in IL-4-stimulated DND39 cells (data not shown).

IRS-1/2 generally plays an important role in the activation of PI3-kinase. However, it is unlikely that Shc is involved in the IL-4-induced PI3-kinase activation, because Shc does not contain the conserved YXXM motif to which the SH2 domain of PI3-kinase binds (38). In this context, Izuhara *et al.* reported that FES can mediate the IL-4-induced PI3-kinase activation, independently of IRS-1/2. Another signaling event induced by IL-4 is IP_3 generation, which is observed in human B cells, but not in murine B cells (30, 39, 40). In DND39 cells, IL-4 induced not only the generation of IP_3 but also the association of Shc with PLCγ1. This association is constitutive and stimulation-independent, because equal amounts of Shc were detected in anti-PLCγ1 immunoprecipitates from unstimulated and IL-4-stimulated DND39 cells. In addition, GST fusion protein containing the SH2–SH2–SH3 region of PLCγ1 precipitated equal amounts of Shc from both the unstimulated and the IL-4-stimulated cells. It is unlikely that PLCγ1 binds to the SH2 domain of Shc, because the enzyme was not tyrosine-phosphorylated in the cells. As an alternative mechanism for protein-protein interaction, the association between a proline-rich motif and the SH3 domain has been proposed (41, 42), and this mechanism could be stimulation-independent. Thus, it is possible that proline-rich region in the CH domain of Shc directly interacts with the SH3 domain of PLCγ1.

The detection of Shc in anti-IL-4Rα immunoprecipitates from IL-4-stimulated DND39 cells indicates that the Shc/PLCγ1 complex can bind to the IL-4Rα via the tyrosine phosphorylation of the IL-4Rα, thereby enables the translocation of PLCγ1 to the plasma membrane where its substrate, phosphatidylinositol 4,5-bisphosphate, is available. Such a mechanism might compensate for the lack of tyrosine phosphorylation of PLCγ1 in IL-4-stimulated DND39 cells, because the function of phosphotyrosine residue of PLCγ1 can be thought to bind with the SH2 domain of adaptor protein and link the enzyme to the plasma membrane. This notion is supported by the fact that the mutation of an essential tyrosine residue of PLCγ1 does not affect catalytic activity of the enzyme (43). Moreover, the activation of PLCγ1 without its direct tyrosine phosphorylation has also been found in a human B cell line and cultured mast cells stimulated with ligation of CD19 and crosslinking of FcϵRI, respectively (44, 45). PLCγ1 activation may lead to the activation of some isoforms of protein kinase C (PKC) in DND39 cells, which is consistent with the fact that IL-4-induced germline ϵ transcription is enhanced by PMA and is inhibited by a PKC inhibitor, K252a (24, 26).

In conclusion, our results show that Shc functions as an adaptor molecule in IL-4 signaling of a human B cell line, DND39. It is important to compare the tyrosine phosphorylation of Shc and that of IRS-1 and IRS-2 in many cell types for a better understanding of the relative importance of these molecules in IL-4 signaling.

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