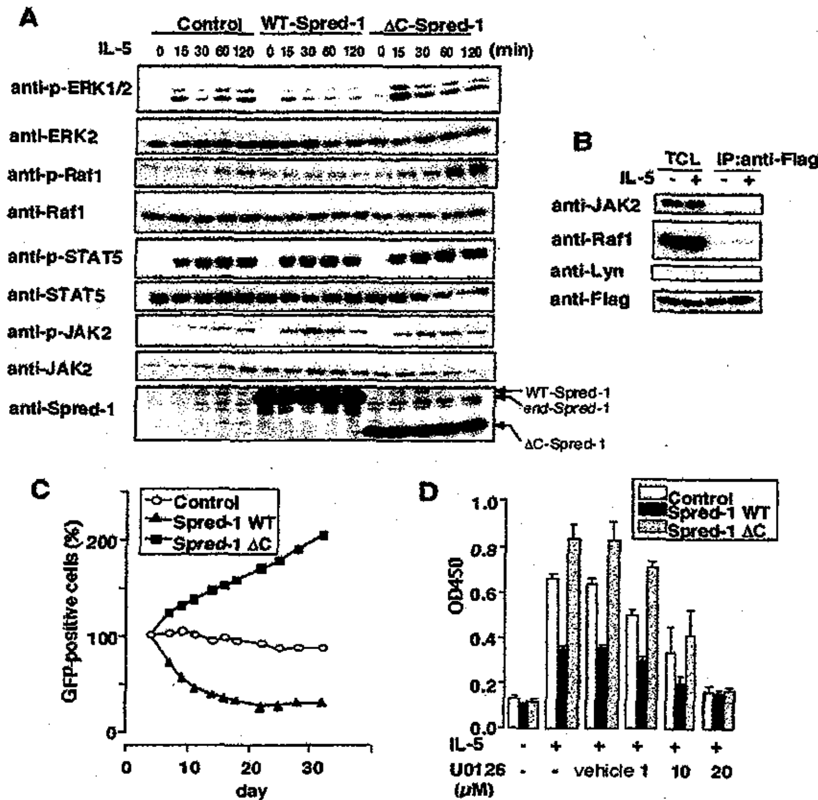

Figure 6. **Effect of WT and ΔC Spred-1 on IL-5–induced ERK activation and proliferation of Y16 cells.** (A) Y16 cells transfected with Flag-tagged WT or ΔC Spred-1 were stimulated with 50 U/ml IL-5 for indicated periods. Cell extracts were immunoblotted with the indicated antibodies. (B) Interaction of Spred-1 with Raf1, Lyn, and JAK2. Y16 cells were transfected with WT Flag-Spred-1. Anti-Flag immunoprecipitates (IP) from Y16 cells with or without incubation of 50 U/ml IL-5 were blotted (IB) with the indicated antibodies. TCL, total cell lysates. (C) Retroviruses carrying Flag-tagged WT and COOH-terminal-truncated Spred-1 (ΔC-Spred-1). cDNAs in pMY-IRES-GFP vector were produced by transfection of the PLAT-E packaging cell line. Y16 cells were infected with viruses for 24 h in the presence of 5 U/ml IL-5. The changes of the proportion of GFP-positive cells with 5 U/ml IL-5 for indicated periods were assayed by flow cytometer. (D) Effect of MEK inhibitor, U0126, on IL-5–induced proliferation. Y16 cells transfected with WT or ΔC Spred-1 were preincubated with buffer, vehicle DMSO, or U0126 for 30 min and cultured with 50 U/ml IL-5 for 48 h. Cell proliferation was measured by colorimetric assay using WST-8 and 1-methoxy PMS.

proposal that the effect of Spred-1 is specific to the downstream of JAK2 (i.e., the Ras–Raf–ERK pathway).

Next, we examined the effect of WT and ΔC-Spred-1 on IL-5–dependent proliferation of Y16 cells. WT and ΔC-Spred-1 cDNAs were introduced into Y16 cells with enhanced GFP (EGFP) using a bicistronic retrovirus vector pMY-IRES-EGFP (35). Because the infected cells expressed both EGFP and Myc-tagged Spred-1, the percentage of infected cells was determined as the EGFP-positive rate by flow cytometry. When Y16 cells were cultured in a medium containing 5 U/ml IL-5 for 4 wk, the proportion of control IRES-EGFP–infected cells was unchanged; however, the population of WT Spred-1–infected cells decreased, whereas that of ΔC-Spred-1–infected cells increased (Fig. 6 C). The MEK inhibitor, U0126, blocked IL-5–induced proliferation of ΔC-Spred-1–transfected Y16 cells as efficiently as parental Y16 cells (Fig. 6 D). These results suggest that the inactivation of Spred-1 enhances IL-5 responses through augmenting Ras–MAPK activity.

To further confirm the effect of Spred-1 on IL-5–dependent signals in more physiological conditions, BM–derived eosinophil (BMEo) cells were analyzed (Fig. 7). Activations of Raf-1 and ERK2 were augmented in Spred-1$^{-/-}$ BMEos compared with WT BMEos, but JAK2 activation was not affected (Fig. 7). These results further supported that Spred-1 inhibits the Ras–ERK pathway downstream of IL-5/JAK2 and that the hyperresponsiveness of Spred-1$^{-/-}$ eosinophils to IL-5 may be explained by enhanced ERK activation.

DISCUSSION

We have recently identified Spreds and determined that Spred-1 interacts with Ras and inhibits growth factor–induced Raf kinase activation (17). The present paper demonstrated that Spred-1 inactivation by a dominant negative mutant enhances IL-5 signals via the Ras–MAPK pathway and IL-5–induced cell proliferation, whereas Spred-1 overexpression suppresses IL-5–induced Ras–MAPK activity. Spred-1–deficient mice exhibit augmented eosinophilia

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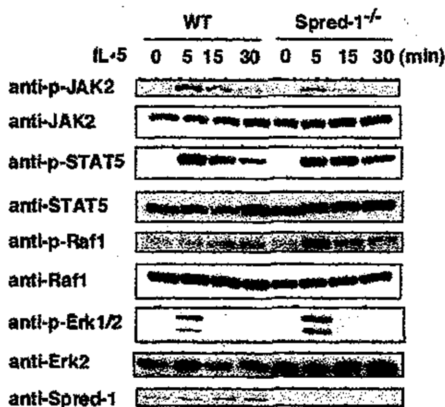

Figure 7. The activation of Raf-1, ERK2, and JAK2 in IL-5–stimulated eosinophils from Spred-1$^{-/-}$ and WT mice. Eosinophils derived from bone marrow were stimulated with 1,500 U/ml IL-5 for indicated periods. Cell extracts were immunoblotted with the indicated antibodies.

in response to IL-5. It has been suggested that the Ras–MAPK pathway might regulate Th cell development (19–21, 36). By using mouse models, we demonstrated that Spred-1 inactivation increases allergen-induced expression of IL-13 and magnifies asthma phenotypes without affecting Th2 differentiation including IL-4 or IgE levels. We further demonstrated that IL-13 production by airway eosinophils is upregulated in Spred-1–deficient mice after allergen challenges. IL-5 (25–27) and IL-13 (28, 29) are critical for the development of asthma. Therefore, these data suggest that Spred-1 plays an important regulatory role in asthma by modulating the signaling of a limited repertoire of Th2 cytokines.

IL-4 has been suggested to be important for the generation of allergen-specific Th2 cells during sensitization (37), and the roles of Ras–MAPK activation in IL-4 production and Th2 differentiation have been investigated. Although some reported no effect on IL-4 production in T cells when inhibiting ERK activity (37), others report a decrease (19, 38) or increase in IL-4 expression and subsequent Th2 differentiation (20, 21). In the present paper, we showed that IL-4 and IFN-γ production in vitro from Th cells in response to anti-TCR from Spred-1–deficient mice is normal. Furthermore, the levels of IL-4 in the airways and serum IgE after allergen challenge in vivo in Spred-1–deficient mice were comparable to those in WT mice. Thus, we consider that Spred-1 may not influence Th2 differentiation.

The higher numbers of IL-5–dependent colonies from the bone marrow and spleen from Spred-1$^{-/-}$ mice than those from WT mice suggest that Spred-1–deficient mice contain an increased number of eosinophil progenitors. The mechanism for this is not clear at present. Our paper shows that Spred-1 inactivation by a dominant negative mutant enhances IL-5 signals via the Ras–MAPK pathway and IL-5–induced cell proliferation using the IL-5–dependent murine B cell line, Y16. Therefore, Spred-deficient eosinophil progenitor cells may respond more efficiently to IL-5. However, we could not rule out the possibility that Spred-1–deficient mice contain a higher number of stem cells, which respond better to the stem cell factor. Allergen-induced eosinophilia is probably due to a higher response of progenitors to IL-5. IL-5 activates several kinases, including Btk, JAK2, Lyn, and Raf-1, as well as the phosphatase SHP2 (32, 39, 40). In eosinophils, JAK2 and Lyn appear to be important for cell proliferation and survival, whereas Raf-1 seems to play a central role in regulating cell function, such as degranulation, adhesion, and survival (41). Because IL-5–induced eosinophilia is markedly enhanced and prolonged in Spred-1–deficient mice, Spred-1 might affect eosinophil proliferation and/or survival in response to IL-5.

Our present data suggest that Spred-1 regulates IL-13 expression through the inactivation of Ras–MAPK signals. This hypothesis is supported by the previous findings that eosinophils have the potential to produce IL-13 (24, 42) and that the Ras–ERK pathway mediates induction of IL-13 expression in T cells (43) and mast cells (44). The downstream functional target molecules of Ras–MAPK in IL-13 induction in response to IL-5 have not been clarified. It has been reported that the IL-5 receptor expression on airway eosinophils is down-regulated after an inhaled allergen challenge and that this is associated with a loss of IL-5 responsiveness (45, 46). However, it is unlikely that Spred-1 modulates IL-5 receptor expression because a specific inhibitor of MAPK kinase (MEK) 1 had no effect on the down-regulation of IL-5 receptor α-chain in eosinophils (47).

We demonstrated that the IL-5–mediated proliferation of Y16 cells is inhibited by WT Spred-1 transfection, whereas it is increased by ΔC-Spred-1 in the present paper. Furthermore, a MEK inhibitor, U0126, blocked IL-5–induced proliferation in Y16 cells, and the inhibitory effect of U0126 was similar between parental Y16 cells and ΔC-Spred-1 transfectants. These data suggest that Spred-1 regulates IL-5–dependent proliferation by modulating the ERK pathway. It has already been demonstrated that pharmacological inhibitor of ERK phosphorylation attenuates allergen-induced airway reactions (48). Our findings and the previous paper suggest that the Ras–ERK pathway is critical in the development of eosinophilic inflammation and AHR.

Given the variety of cellular distribution of Spred-1 expression including airway epithelial cells, endothelial cells (49), hematopoietic progenitor cells (unpublished data), and eosinophils in the present paper, it is unlikely that all of the asthma phenotypes in Spred-1$^{-/-}$ mice are due to Spred-1 deficiency on eosinophils. In addition, other Spred family members might also regulate eosinophilia and allergen-induced AHR. However, our data clearly demonstrate that Spred-1$^{-/-}$ mice showed prominent airway eosinophilia and intact Th1/Th2 differentiation, and Spred-1$^{-/-}$ eosinophils are more sensitive to IL-5–induced proliferation and Raf-1/ERK activation, as well as eotaxin-induced migration in vitro. Therefore, it is possible that hyperresponsive eosinophils to IL-5 and eotaxin in Spred-1$^{-/-}$ mice may predominantly contribute to the airway pathology in these mice.

Our studies provide evidence that Spred-1 is critical for the IL-5 response and IL-13 production and for allergen-

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induced asthma without influencing Th2 development. Although the relationship between total IgE and asthma prevalence is well known (50), an atopic status is not an associated finding in severe asthma (51). There is considerable evidence to support a critical role for the involvement of Th2 cytokines and eosinophils both in atopic and in nonatopic asthma. In particular, IL-5 is critical for eosinophilia, and IL-13 has the potential to modulate airway inflammation and AHR independently of IgE in animal models. Therefore, we propose that Spred-1 may be a useful therapeutic target to compensate for atopic and nonatopic asthma.

MATERIALS AND METHODS

Mice. A genomic library from the 129/SV mouse strain (Stratagene) was screened with a cDNA probe of the mouse Spred-1, and several overlapping positive clones, including from second to fourth exons, were identified. The targeting vector was constructed by replacing the fourth exon with a pgk-neo cassette while preserving 5-Kb (left arm) and 3-Kb (right arm) flanks of homologous sequences (Fig. 1 A). The hsv-TK gene was inserted for negative selection. Homologous recombination in murine embryonic stem cells was performed as described previously (52) and was confirmed by Southern blot analysis. The chimeric mice were backcrossed to C57BL/6 three times. The resultant F3 mice were intercrossed to obtain the offspring for analysis. Genomic PCR was performed as described previously (53). The following primer sets were used: Spred-1 WT, 5′-GCAGACTACAGACATCCGGACATGTGG-3′ and 5′-CGCATGGCCCCAATGATACCGGCAAG-3′; Spred-$1^{-/-}$, 5′-CGAGATCAGCAGCCTCTGTTCCACATAC-3′ and 5′-CCAAGAGAGCTGAGGATGAACTCACCG-3′.

RT-PCR. To detect Spred-1, total RNA was extracted from the bone marrow, spleen, and brain using TRIzol reagent (GIBCO BRL). RT-PCR was performed using a GeneAmp RNA PCR kit (PE Biosystems) according to the manufacturer's instructions. The specific primers for Spred-1 were 5′-GATGAGCGAGGAGACGGCGAC-3′ and 5′-GTCTCTGAGTCTCTCTCCACGGA-3′.

Sensitization and challenge. 10–12-wk-old C57BL/6 mice, Spred-$1^{-/-}$ mice, or their WT littermates were sensitized with intraperitoneal injections of 20 μg OVA (Grade V; Sigma-Aldrich) plus 2.25 mg aluminum hydroxide (Pierce Chemical Co.) on days 1 and 14. On days 26–28, mice received aerosol challenge containing either saline or 1% OVA for 20 min/d.

Measurement of airway responsiveness. On day 30, 36 h after the last aerosol challenge, mice were ventilated to measure AHR to acetylcholine aerosol as described previously (22, 54). Airway opening pressure was measured with a differential pressure transducer and continuously recorded. Stepwise increases in the acetylcholine dose were given with an ultrasonic nebulizer. All animal experiments were approved by the Committee on Animal Research, Faculty of Medicine, Kyushu University. The data were expressed as the provocative concentration 200 (PC_{200}), the concentration at which airway pressure was 200% of its baseline value, and PC_{200} was calculated by log-linear interpolation for individual mice. Lower log PC_{200} values represent greater AHR. The serum levels of total and OVA-specific IgE were analyzed by ELISA with rat anti–mouse IgE (Serotec Ltd.).

BAL and cytokine measurements. Mice were exsanguinated with a lethal dose of pentobarbital, and their lungs were gently lavaged with 1 ml of 0.9% saline via a tracheal cannula. Total and differential BAL cell counts were performed as described previously (22). Samples were centrifuged at 2,000 revolutions/min for 10 min, and the supernatants were stored at −80°C. Mouse IL-4, IL-5, IL-13, and IFN-γ were quantified using ELISA kits (Biosource International) according to the manufacturer's protocols. Measurement of cytokine production in BAL fluid was assessed by ELISA assays.

Histological assessment. Lungs were fixed with 10% formalin, and tissue sections were stained with Alcian blue/PAS to determine the presence of mycin glycoconjugates (22). The numerical scores for the abundance of PAS-positive mucus-containing cells in each airway were determined as follows: 0, <5% PAS-positive cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75% (36).

Collection of lung cells and lymph node cells. Enzymatic digestion of the lungs was performed with collagenase type 1A, hyaluronidase, and DNase, and the samples were filtered through a 52-μm nylon mesh. The erythrocytes were removed by lysis. Cells were stained with FITC-labeled anti-NK1.1 mAb, PE-labeled anti-CD19 mAb, PerCP-labeled anti-CD3 mAb, and allophycocyanin-labeled anti-CD4 mAb. After flow cytometric analysis using a FACSCalibur with CellQuest software (BD Biosciences), the absolute numbers of lung immune cells were calculated. Draining lymph node cells were collected from paratracheal and mediastinal lymph nodes, and $CD4^+$ T cells isolated with a magnetic cell sorter (Miltenyi Biotec) were stimulated with a combination of 30 μg/ml anti-TCR mAb (H57-597) and 1 μg/ml anti-CD28 mAb (PV-1) for 48 h. ELISA assays for cytokine production were performed as described previously (54).

Cell purification and induction of helper T cells. Naive $CD4^+$ T cells isolated from spleens were stimulated with a combination of anti-TCR mAb and anti-CD28 mAb, and 10 U/ml rIL-12 and anti–IL-4 mAb (11B11) were added for Th1 development, while 100 U/ml rIL-4 and anti–IL-12 mAb (C15.6 and C17.8) were added for Th2 development. ELISA assays for cytokine production and intracellular cytokine staining were performed as described previously (54).

In vitro colony assay. Single cell suspensions were isolated from the bone marrow or spleen of WT and Spred-$1^{-/-}$ mice. The erythrocytes were removed by lysis using NH_4Cl and 3×10^3 cells were plated in methylcellulose (Methocult3434; StemCell Technologies Inc.) containing 10 ng/ml of IL-5. On day 14, the numbers of colonies were counted microscopically.

Isolation of lung eosinophils. BAL was performed in OVA-sensitized and -challenged mice by three repeated lavages with RPMI 1640 containing penicillin-streptomycin at 25 cm H_2O. The lavage fluid was collected and incubated for 30 min to remove macrophages from the cell suspension by adherence to plastic. Eosinophils were isolated by a negative selection strategy, removing B cells and T cells with Ab-conjugated magnetic beads (MACS; Miltenyi Biotec) specific for CD45-R (B220) and CD90 (Thy1.2), respectively. The purity of the recovered eosinophils was confirmed to be >98% by staining cytospin preparations with Diff-Quik (neutrophils comprise the contaminating cell populations). Eosinophils were cultured at 10^6 cells/ml for the indicated times.

IL-5 and IL-13 treatment in vivo. Recombinant murine IL-5 (10,000 U/d) was injected intraperitoneally into WT- or Spred-1–deficient mice for 4 d (30). Differential counts were performed by examination of blood smears stained with a modified Wright-Giemsa stain at various time points.

IL-13 administration was performed as described previously (22). A recombinant murine IL-13 solution (0.5 g) or a vehicle solution was instilled intratracheally on days 1, 3, and 5. BAL was performed on day 6, 24 h after the last instillation, and BAL cell differentials were determined.

Cell lines and cultures. IL-5–dependent murine cell line, Y16 was cultured in an RPMI 1640 medium containing 5% FBS, 50 mM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 5 U/ml IL-5 (55). The retrovirus-packaging cell line PLAT-E was maintained in DMEM containing 10% FBS.

Bone marrow cells from the femoral bone of Spred-$1^{-/-}$ mice or their WT littermates were cultured in RPMI 1640 supplemented with 100 U/ml murine IL-5, 30% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol for 2 wk to generate populations of BMEos that were >95% pure.

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Chemotaxis assay. The chemotaxis assay was performed in a 96-well disposal chemotaxis plate (5-μm pore size; Neuro Probe). In brief, eotaxin was diluted in RPMI 1640 with 0.1% BSA and placed in the bottom wells (27 μl), 25 μl of cell suspension at 4×10^6 cells/ml was placed on the top well of the chamber, which was separated from the bottom well by a polycarbonate filter. The plate was incubated for 60 min at 37°C in a humidified incubator with 5% CO_2. The cells remaining on top of the filters were absorbed off, the filter tops were carefully washed, and the plates were centrifuged to pellet all cells on the undersides of the filters. The filters were removed, and cells in the bottom wells were counted by light microscopy. Data are reported as a migration index, calculated as follows: (number of cells migrating to chemoattractant)/(number of cells migrating to vehicle).

Cell proliferation assay. Cell proliferation was assayed using a Cell Counting Kit (Dojindo). In brief, 10^3 Y16 cells were plated in 96-well plates in RPMI 1640 medium with IL-5 and cultured for 48 h. After adding WST-8 and 1-methoxy PMS, optical density for 450 nm was measured.

Plasmids, transfection, and infection. WT Spred-1 and COOH-terminal–truncated Spred-1 (ΔC-Spred-1) were subcloned into pMY-IRES-GFP vectors for retrovirus infection as described previously (56). In brief, pMY-IRES-GFP vectors were transfected into a PLAT-E packaging cell line using the transfection reagent FuGENE 6 (Boehringer) to obtain the viruses (35). Y16 cells (2×10^5 cells) were infected with viruses on a RetroNectin (TaKaRa)-coated plate for 24 h in the presence of 5 U/ml IL-5. Cells were washed three times with PBS, resuspended in RPMI 1640, 5% FBS containing 5 U/ml IL-5, and incubated for the indicated times. 10^4 cells were analyzed for GFP fluorescence on a flow cytometer.

Immunochemical analysis. Immunoprecipitation and immunoblotting were performed using anti-STAT5, anti-ERK2 (Santa Cruz Biotechnology, Inc.), anti-Flag (M2; Sigma-Aldrich), anti–phospho-TAT5, anti–phospho-ERK2 (Cell Signaling), and anti–phospho-Ser338-Raf-1 (Upstate Biotechnology) antibodies as described previously (56, 57). Anti-Spred-1 antibody was prepared by immunizing rabbits (17).

Statistical analysis. Values were expressed as the mean ± SEM. Differences among groups were analyzed using unpaired Student's *t* tests or an analysis of variance together with a post-hoc Bonferroni analysis. Nonparametric data were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney U test. p-values <0.05 were considered to be significant.

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