Fig. 2. Responses of *lnk*$^{-/-}$, *SH2-B*$^{-/-}$ or *APS*$^{-/-}$ BMMCs (filled bars) and of respective control BMMCs (open bars) induced by activation of c-Kit or FcεRI. (A) Proliferation upon stimulation with various concentrations of SCF. Values shown are the mean cpm ± SD of triplicate determinations. (B) Adhesion to fibronectin induced by various concentrations of SCF or 10 ng/ml PMA. Shown are average ± SD of triplicate measurements. (C) Degranulation after cross-linking FcεRI. Cells sensitized with anti-DNP IgE mAb were stimulated with the various concentrations of DNP-BSA. Shown is the percentage of β-hexosaminidase activity released into culture supernatants out of the total β-hexosaminidase initially stored in cells. *APS*$^{-/-}$ BMMCs showed augmented degranulation responses (see also Table 1). Representative results of three independent experiments are shown from (A) through (C).

mediated by binding of monomeric IgE to FcεRI was also comparable (Fig. 3C). Tyrosine phosphorylation of various cellular proteins was rapidly induced after cross-linking FcεRI in mast cells and was comparable between

APS$^{-/-}$ and wild-type BMMCs. Phosphorylation of neither Akt nor PKCδ molecules was affected in the absence of APS (data not shown).

Decreased actin assembly in *APS*$^{-/-}$ BMMCs

It has been shown that Lnk associates with an actin binding protein ABP-280 [19] and that SH2-B plays a role in actin reorganization and cell motility mediated by growth hormone receptor [20,21]. We recently found that Lnk facilitates actin reorganization in transfected fibroblast cells (S.M.K. and S.T., unpublished data). In addition, a negative correlation between actin polymerization and FcεRI-mediated degranulation from RBL-2H3 mast cell line has been presented [22,23].

We speculated APS may regulate actin cytoskeleton, which potentially has regulatory process for degranulation in mast cells. Therefore, we investigated consequences of inhibition of actin polymerization induced by cross-linking FcεRI in BMMCs and its effect on degranulation by treatment with latrunculin. Treatment of sensitized BMMCs with latrunculin resulted in the reduction of F-actin contents as demonstrated by rhodamine–phalloidine binding (Fig. 4A, left panel). Cross-linking FcεRI induced reduction of F-actin contents in stimulated BMMCs. Consistent with observations using RBL-2H3 cells, inhibition of actin assembly by treatment with latrunculin enhanced degranulation from normal BMMCs in a dose-dependent manner (Fig. 4A, right). Interestingly, sensitized *APS*$^{-/-}$ BMMCs showed reduced F-actin content (about 70% of control) compared to wild-type cells (Fig. 4B, left). The reduction in F-actin contents became less evident in cells treated with latrunculin. Finally, the effect of latrunculin on degranulation was compared between *APS*$^{-/-}$ and control BMMCs. As shown in Fig. 4B, augmented degranulation by *APS*$^{-/-}$ BMMCs became less evident by treatment with latrunculin, which was well correlated with difference in F-actin contents between latrunculin treated *APS*$^{-/-}$ and control cells. These results suggested that *APS*-deficiency in mast cells made actin assembly at relatively low levels and that resulted in facilitated degranulation process after cross-linking FcεRI.

Table 1
Enhancement of FcεRI-induced degranulation in *APS*$^{-/-}$ BMMC

DNP-BSA(μg/ml)	Degranulation (% maximal response induced by PMA plus ionomycin)			
	0	0.1	0.5	2.5
+/+ (*n* = 11)	5.6 ± 0.9	19.4 ± 2.4	28.9 ± 2.5	29.4 ± 2.1
−/− (*n* = 11)	5.0 ± 0.7	25.8 ± 3.9	39.5 ± 3.9*	40.6 ± 3.4**
(% +/+ response)	(89%)	(133%)	(136%)	(138%)

Sensitized BMMCs were stimulated with the various concentrations of DNP-BSA or 10 ng/ml PMA plus 400 ng/ml ionomycin. Values represent the mean ± SE of % β-hexosaminidase activity normalized by the value induced with PMA plus ionomycin as 100%. *$p < 0.05$, **$p < 0.01$ compared to +/+ BMMCs by Student's *t* test.
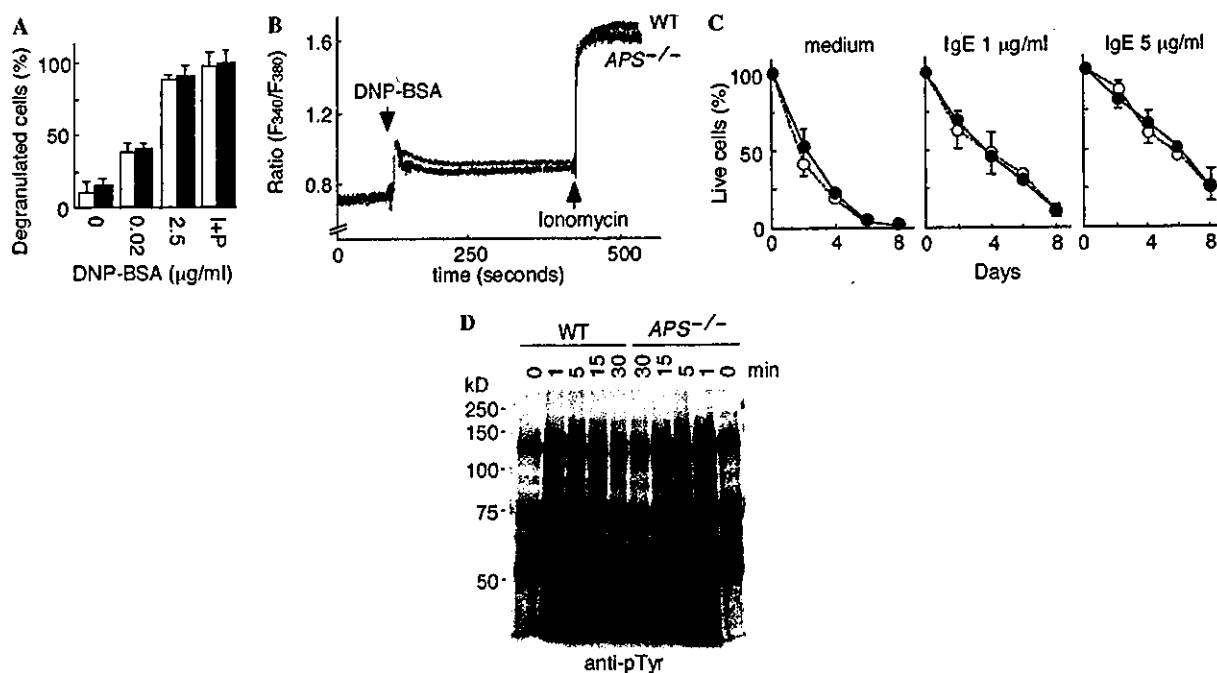
Fig. 3. Cellular responses of $APS^{-/-}$ BMMCs mediated through cross-linking FcεRI. (A) Proportion of degranulated cells after cross-linking FcεRI with various concentrations of antigens was determined by cytochemistry. Percentages of degranulated cells were comparable between $APS^{-/-}$ (closed bars) and wild-type control mice (open bars). The average ± SD of three independent experiments are shown. (B) Calcium influx induced upon cross-linking FcεRI in $APS^{-/-}$ (lower line) and wild-type (upper line) BMMCs. After IgE sensitization, BMMCs were loaded with Fura PE3 and stimulated with 5 μg/ml DNP-BSA and 10 μg/ml ionomycin at the indicated time points (arrows), and fluorescence intensity ratio at 340–380 nm was measured. Representative results of two independent experiments are shown. (C) Survival of $APS^{-/-}$ (closed circles) and wild-type (open circles) BMMCs by binding of monomeric IgE to FcεRI. Cells were cultivated in the absence or in the presence of various concentrations of monomeric IgE and percentages of live cells were measured. The average ± SD of three independent experiments are shown. (D) Tyrosine phosphorylation of total cellular proteins after cross-linking FcεRI. Sensitized BMMCs were stimulated with 2.5 μg/ml DNP-BSA for the indicated times. Total cell lysates were separated through SDS–PAGE and subjected to immunoblot using anti-phosphotyrosine mAb (4G10). Representative results of three experiments are shown.

Discussion

We investigated functions of Lnk, SH2-B or APS in mast cells, since possible regulatory roles of Lnk-family adaptor proteins in signaling through c-Kit or FcεRI had been suggested. We established BMMCs lacking either Lnk, SH2-B or APS and examined their cellular responses. None of those mutant BMMCs showed altered responses against IL-3 or SCF, the c-Kit ligand. *APS*-deficiency resulted in enhanced FcεRI-mediated degranulation, while both $Ink^{-/-}$ and $SH2$-$B^{-/-}$ BMMCs did not show any abnormal responses induced by cross-linking FcεRI.

We have shown that Lnk negatively regulates c-Kit signaling in B cell precursors and hematopoietic progenitor cells [8,10]. We did not observe significant enhancement in SCF-dependent growth of $Ink^{-/-}$ BMMCs in contrast to a previous report by Velazquez et al. [11]. SCF-dependent adherence was also comparable to normal cells. Expression levels of *Ink* transcripts are rather low in BMMCs compared to B-lineage cells or hematopoietic progenitor cells (un-

published data). It is likely that *Ink*-deficiency alone hardly affects mast cell function because of low expression of Lnk in mast cells.

APS had been cloned as a possible candidate substrate for the c-Kit [7]. However, $APS^{-/-}$ BMMCs did not show any altered responses upon stimulation with SCF. Instead, they showed enhanced FcεRI-mediated degranulation. $APS^{-/-}$ BMMCs showed reduced actin assembly at steady state compared to normal BMMCs. Inhibition of actin assembly in normal BMMCs by latrunculin resulted in enhanced degranulation similar to $APS^{-/-}$ BMMCs. In $APS^{-/-}$ mice, B-1 cells in peritoneal cavity increased and showed reduced F-actin contents. Conversely, in transgenic mice overexpressing APS in lymphocytes, B cells were reduced and showed enhanced actin assembly [17]. These results suggest that APS may negatively regulate degranulation process by controlling actin dynamics in mast cells. In RBL-2H3 mast cells, F-actin assembly induced by cross-linking FcεRI negatively controls degranulation as well as calcium signaling [22,23]. Oka et al. [24] recently reported that monomeric IgE binding induced actin assembly and that inhibition
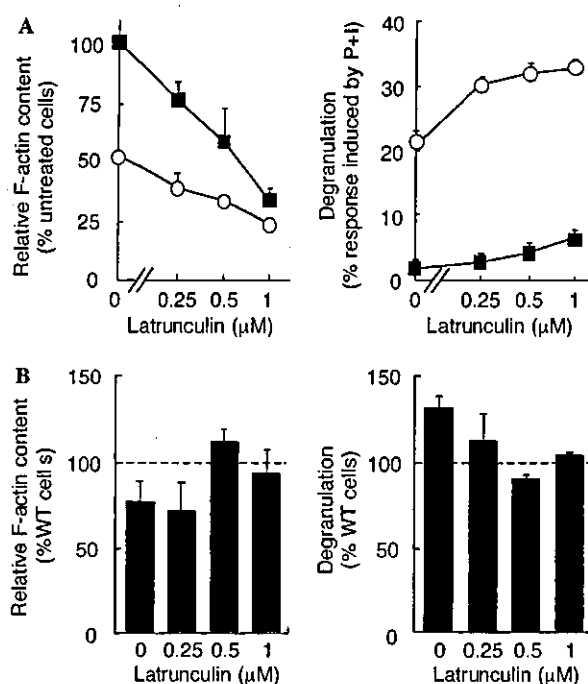
Fig. 4. Enhanced degranulation correlated with reduced F-actin contents in BMMCs treated with inhibitor of actin assembly, latrunculin or by APS-deficiency. (A) Treatment with latrunculin inhibited actin assembly and resulted in reduced F-actin content in BMMCs. Sensitized wild-type BMMCs were incubated with the various concentrations of latrunculin, kept unstimulated (squares) or stimulated with 2.5 μg/ml DNP-BSA (circles). F-actin contents of cells were then analyzed by rhodamine–phalloidin staining and flow cytometry, and the results are shown as relative F-actin contents compared with that of unstimulated cells in the absence of latrunculin (left). Degranulation was determined by measuring β-hexosaminidase activity released into culture supernatants, and results were shown as percent maximal responses induced by PMA and ionomycin treatment (right). (B) F-actin content of APS$^{-/-}$ BMMCs in the absence or the presence of various concentrations of latrunculin was measured and relative F-actin contents compared with those of control cells treated with the same concentrations of latrunculin were shown (left). Degranulation from APS$^{-/-}$ BMMCs treated with latrunculin was measured, and shown as percent reaction compared with those from wild-type control cells in the same conditions (right). Results shown are means ± SE of values obtained from three independent experiments.

of IgE-induced actin assembly by cytochalasin D initiates calcium influx and degranulation. Although enhancement of calcium influx in APS$^{-/-}$ BMMCs was not observed, reduction of actin assembly in APS$^{-/-}$ BMMCs may lead to augmented degranulation in analogy with those observed in RBL-2H3 mast cells. The molecular mechanisms for APS-mediated actin assembly as well as APS function downstream of cross-linking FcεRI remain to be elucidated.

APS function in insulin-R signaling has been also indicated in various experiments using cell lines [15,16,25–27]. APS$^{-/-}$ mice exhibited increased sensitivity to insulin and enhanced glucose tolerance [28]. It is intriguing to examine whether effect of APS-deficiency

on insulin sensitivity is also mediated by actin dynamics. Regulation of actin cytoskeleton seems one of the common functions of Lnk-family adaptor proteins. Lnk associates with an actin binding protein ABP-280 [19] and facilitates actin assembly in overexpressed fibroblasts by activating Vav and Rac (S.M.K. and S.T., unpublished data). SH2-B is required for actin reorganization and regulates cell motility induced by GH-R activation [20,21].

SH2-B has been identified as a possible adaptor binding to ITAMs of FcεRI γ chain [6]. However, all examined responses induced by FcεRI ligation were normal with SH2-B$^{-/-}$ BMMCs. It seems SH2-B-deficiency do not affect mast cell function. However, it should be notified that interaction of SH2 domains of Lnk-family proteins with c-Kit or ITAM of FcεRI γ chain had been demonstrated in overexpression systems with different combinations, for example, SH2-B with FcεRI γ chain, APS with c-Kit. SH2-B$^{-/-}$ mice showed mild growth retardation and infertility due to impaired maturation of gonad organs [12]. Thus, SH2-B seemed to have a true target except FcεRI, worked as a positive regulator of signal transduction in contrast to Lnk and APS that function as negative regulators as shown in previous studies and in this study. Despite the significant structural similarities between APS, Lnk, and SH2-B, their functions appear to be quite different from each other. However, possible common functions of those adaptor proteins in vivo should be examined by generating mutant mice lacking APS, Lnk or SH2-B in various combinations.

In conclusion, our studies describe roles of Lnk family adaptor proteins on BMMCs. Both Lnk and SH2-B were dispensable for various mast cell responses mediated through c-Kit, FcεRI as well as IL-3-R. APS plays a role in controlling FcεRI-induced degranulation response but not in c-Kit-mediated proliferation or adhesion. APS may regulate degranulation by controlling actin dynamics in mast cells.

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"Homing to Niche," a New Criterion for Hematopoietic Stem Cells?

By combining cell surface staining with fluorochrome-conjugated monoclonal antibodies and Hoechst 33342 dye supravital staining, Matsuzaki et al. have succeeded in enriching hematopoietic stem cells (HSCs) essentially to homogeneity. When single-cell transplantation analysis was performed using the isolated cells, over 95% of the recipient mice showed long-term multilineage engraftment. The work demonstrates unexpectedly high marrow seeding efficiency of HSCs and proposes high marrow homing capacity as a new criterion for HSCs.

Stem cells are generally defined as cells capable of both self-renewal and multilineage differentiation. During development and regeneration of a given tissue, such cells give rise to non-self-renewing progenitors with restricted differentiation potential, and finally to functionally mature cells, while maintaining primitive stem cells. Because of these unique properties, stem cells offer the novel and exciting possibility of organ reconstitution in place of transplanted or artificial organs in the treatment of organ failure. Among different stem cells, hematopoietic stem cells (HSCs) are the best studied.

Purification of HSCs has progressed significantly over the last two decades, strongly assisted by well-established assay techniques. Technological advances in flow cytometry and electronic cell sorting have also aided in stem cell purification. The frequency of HSCs in sorted cells is determined using in vivo long-term marrow reconstitution assays, counting competitive repopulation units (CRUs) by limiting-dilution analysis. Purity of HSCs in the sorted fraction is estimated by dividing CRU frequency by seeding efficiency, a probability representing homing of tail-vein-infused HSCs to the bone marrow. The seeding efficiency of HSCs, like that of CFU-S (Siminovitch et al., 1963), was estimated to be between 10% and 20%, based on the 48 hr bone marrow seeding of cells capable of long-term reconstitution (Lanzkron et al., 1999). Using seeding efficiency as a "fudge factor," previous reports tended to overestimate the purity of HSCs in a given fraction. In 1996, however, Osawa et al. showed that transplantation of single CD34$^{low/negative}$Kit$^+$Sca$^+$Lin$^-$ (CD34$^-$KSL) cells into lethally irradiated congenic mice resulted in engraftment of 20% of the recipients, clearly indicating that the seeding efficiency must be higher than 20%. Single-cell transplantation experiments subsequently demonstrated engraftment rates of 30%–40% (Ema et al., 2000; Wagers et al., 2002), but the actual seeding efficiency of HSCs remained unclear.

In this issue, Matsuzaki et al. have demonstrated absolute engraftment of lethally irradiated syngenic recipients transplanted with sorted single HSCs from GFP-transgenic mice. While this study can be regarded as only another lap around the HSC-purification racetrack, it is a remarkable achievement, demonstrating over 95% long-term multilineage reconstitution after transplantation of a single purified HSC. The key to this success is six-color FACS analysis and cell sorting, using a combination of cell surface staining with monoclonal antibodies and Hoechst 33342 dye efflux analysis. The cells that have the strongest dye efflux capacity (Tip-SP cells), with a CD34$^-$KSL phenotype, are HSCs with nearly 100% long-term marrow reconstitution capacity after single cell transplantation. One of three sets of experiments revealed long-term multilineage reconstitution in all 33 recipients after transplantation. The study proves that the marrow seeding efficiency of HSCs is nearly 100%.

These results highlight some intriguing features of HSCs. The efficiency of HSCs (among Tip-SP CD34$^-$KSL cells) in seeding their bone marrow niche was nearly 100%, consistent with recent observations made by Iscove et al. (Benveniste et al., 2003). Based on these results, Matsuzaki et al. propose the capacity to home to bone marrow as a third criterion in defining HSCs. However, it may be too early to include homing capacity among the criteria for HSCs since it is not yet clear whether Tip-SP CD34$^-$KSL cells constitute all HSCs in bone marrow. HSCs may exist that lack homing capacity and whose stem cell activity can be tested only by intrabone marrow injection. Perhaps HSCs and early hematopoietic progenitor cells differ simply in their ability to home to a bone marrow niche where HSCs can self-renew. This unexpectedly high homing capacity of HSCs is a significant and intriguing aspect of stem cell biology. Although osteoblastic cells reportedly have a role in the HSC bone marrow niche (Calvi et al., 2003; Zhang et al., 2003), not much is known about the site to which HSCs home. The molecular mechanisms regulating HSC homing deserve more intensive study, especially given the importance of such homing in a variety of medical applications.

The results here also may provide new insights into the debate about stem cell fate. Both stochastic and deterministic models have been proposed for the alternative fates of self-renewal versus lineage commitment. Till et al. suggested that stem cell fate is determined stochastically with an average frequency of ~60% for self-renewal (Till et al., 1964; Vogel et al., 1968). The findings of Matsuzaki et al. do not support this model; they favor the nonrandom, deterministic model. Because each transplanted single HSC reconstituted the recipient's bone marrow long-term, every individual HSC must have taken the path of self-renewal at the first cell division. Presumably, external signals promote this self-renewal.

The data of Matsuzaki et al. also implicate the cells with highest dye efflux capacity as the most primitive HSCs. Although Tip-SP CD34$^-$KSL cells may not include all HSCs in the bone marrow, they represent a selected population for pure HSCs. Thus, at a minimum these

findings suggest a relationship among dye efflux capacity, repopulating capacity, and stem cell homing. Whether these stem cell functions are related to bcrp-1 (Zhou et al., 2001), a transporter responsible for the SP phenotype in HSCs and possibly other adult stem cells, poses a challenging question for stem cell biology.

Matsuzaki et al. have not only shown us how to isolate pure HSCs, which should aid studies of cell fate decision and stem cell regulation. They have provided important clues to understanding stem cell homing and the HSC niche.

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