Dysregulation of Src Family Kinases in Mast Cells from Epilepsy-Resistant ASK versus Epilepsy-Prone EL Mice[1]

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EL mice have been used as a model of epilepsy, whereas ASK mice are an epilepsy-resistant variant originating from a colony of EL mice. Mast cell-dependent anaphylaxis is easily inducible by stimulation with IgE and Ag in ASK mice, whereas EL mice are resistant to such stimuli. In this study we have characterized mast cells derived from these two strains. ASK mast cells proliferated more vigorously than EL cells in response to IL-3 and stem cell factor. Although ASK mast cells degranulated less vigorously than EL mast cells upon stimulation with IgE and Ag, ASK cells produced and secreted several-fold more TNF-α and IL-2 than EL cells. Consistent with the similarities of these ASK and EL mast cell responses with phenotypes of *lyn*$^{-/-}$ and wild-type mast cells, respectively, Lyn activity was reduced in ASK cells. In addition to the impaired Lyn activity, ASK cells just like *lyn*$^{-/-}$ cells exhibited reduced Syk activity, prolonged activation of ERK and JNK, and enhanced activation of Akt. Furthermore, the lipid raft-resident transmembrane adaptor protein Cbp/PAG that associates with Lyn was hypophosphorylated in ASK cells. Importantly, similar to *lyn*$^{-/-}$ cells, Fyn was hyperactivated in ASK cells. Therefore, these results are consistent with the notion that Lyn-dependent phosphorylation of Cbp/PAG negatively regulates Src family kinases. This study also suggests that reduced activity of Lyn, a negative regulator of mast cell activation, underlies the susceptibility of ASK mice to anaphylaxis and implies that dysregulation of Lyn and other Src family kinases contributes to epileptogenesis. ***The Journal of Immunology,*** **2007, 178: 455–462.**

Mast cells are the crucial effector cell type for IgE-dependent immediate hypersensitivity and allergic diseases (1). These allergic reactions are mediated by mast cell activation through the high-affinity IgE receptor, FcεRI. FcεRI consists of an IgE-binding α subunit, a four-transmembrane β subunit with receptor-stabilizing and signal-amplifying functions, and two disulfide-bonded, signal-generating γ subunits (2). Upon receptor aggregation, FcεRI β subunit-associated Lyn, an Src family protein tyrosine kinase (PTK),[4] phosphorylates tyrosine residues of the ITAM in β and γ subunits. Phosphorylated ITAM in β and γ subunits recruit Lyn and Syk molecules, respectively. These ITAM-bound PTK are activated and phosphorylate a multitude of signaling proteins, leading to the activation of several signaling pathways including PI3K, phospholipase C/Ca^{2+}, and MAPK (3, 4). In addition to Lyn, recent studies suggested the presence of another pathway required for degranulation: Fyn, another FcεRI-associated Src family PTK (SFK), mediates phosphorylation of an adaptor protein Gab2 leading to PI3K activation (5) as well as Ca^{2+}-independent microtubule formation via the activation of RhoA (6). Concerted action of these pathways leads to degranulation (release of preformed vasoactive amines, proteases, and other proinflammatory mediators), synthesis and release of leukotrienes and their derivatives, and production and secretion of cytokines and chemokines.

SFK are strictly regulated by the phosphorylation of two tyrosine residues: autophosphorylation of the tyrosine residue in the activation loop of the catalytic domain is required for full activation, whereas phosphorylation of the tyrosine residue (Tyr^{529} in mammalian c-Src) in the C-terminal region abolishes activity (7). Phosphorylation of this negative regulatory residue is mediated by another PTK, C-terminal Src kinase (Csk) (8). Csk is a cytoplasmic PTK and must be recruited to the plasma membrane to phosphorylate membrane-bound SFK. The transmembrane adaptor protein, termed Csk-binding protein (Cbp), or phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), binds to the Src homology 2 domain of Csk (9, 10). Cbp/PAG localized to lipid rafts interacts with Csk through the phosphorylated Tyr^{314} (in mouse and rat Cbp/PAG, or Tyr^{317} in the human homolog), which is phosphorylated by SFK. Based on these observations, a feedback regulation model of SFK activity has been proposed in several cell types: activation of SFK by an extracellular stimulus leads to the phosphorylation of Cbp/PAG, resulting in the recruitment of Csk to lipid rafts and is followed by termination of SFK activation (11–14). In contrast, Cbp/PAG is constitutively tyrosine phosphorylated and associated with Csk in resting T cells; stimulation via TCR causes transient dephosphorylation of Cbp/PAG contributing apparently to increased SFK activity needed in early phases of T cell activation (10, 12, 15).

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[4] Abbreviations used in this paper: PTK, protein-tyrosine kinase; SFK, Src family PTK; Csk, C-terminal Src kinase; Cbp, Csk-binding protein; PAG, phosphoprotein associated with glycosphingolipid-enriched microdomain; BMMC, bone marrow-derived mast cell; SCF, stem cell factor; NMDA, glutamate *N*-methyl-D-aspartate; HSA, human serum albumin.

EL mice are an inbred strain extensively studied as a model of human epilepsy or complex partial seizures (16). Convulsion can be induced in EL mice by physical stresses such as rhythmic vestibular stimulation. The major gene loci, *EL-1* and *EL-2*, responsible for this epileptic phenotype was mapped on chromosomes 9 and 2, respectively. ASK mice originated from a colony of EL mice as an epilepsy-resistant variant. Previous studies showed that ASK mice are highly susceptible to IgE-dependent anaphylactic stimuli in contrast with EL mice that are resistant to such stimuli (17, 18). However, molecular basis of the differences in contrasting epileptic and anaphylactic phenotypes in EL and ASK mice has not been known at all. In the present study, we have compared the mast cell phenotype between the two strains. Interestingly, we have found that bone marrow-derived mast cells (BMMC) from ASK mice exhibit a phenotype similar to that of Lyn-deficient mice in comparison with EL mast cells: enhanced proliferative response to IL-3 and stem cell factor (SCF) and a slightly reduced degranulation, but an excessive production of TNF-α and IL-2, in response to FcεRI stimulation. Importantly, analysis of Lyn and other SFK in these mast cells has revealed the presence of a Lyn-dependent, SFK-negative regulatory feedback mechanism by which the reduced Lyn activity in ASK cells eventually leads to overproduction of cytokines.

Materials and Methods

Antibodies

Anti-DNP IgE mAb (19) was purified from ascites of hybridoma-bearing mice as previously described (20). Anti-FcεRI β subunit mAb was a gift from Dr. J. Rivera (National Institutes of Health, Bethesda, MD). Rabbit polyclonal Ab to the cytoplasmic domain of human Cbp/PAG and cross-reactive with the murine homolog and mAb PAG-C1 (reactive with C-terminal peptide of human and murine Cbp/PAG) were previously described (9, 10). Another anti-Cbp/PAG Ab (9) was provided by Dr. M. Okada (Osaka University, Osaka, Japan). Commercial sources of Abs are as follows: anti-phosphotyrosine mAb 4G10 from Upstate Cell Signaling Solutions; anti-Lyn, anti-Syk, anti-Btk, anti-JNK1 (sc-474), anti-p38, anti-Akt, anti-Src, anti-Fyn, and anti-Csk from Santa Cruz Biotechnology; anti-ERK from Zymed Laboratories; anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}), anti-phospho-p38 MAPK (Thr^{180}/Tyr^{182}), anti-phospho-Akt (Ser^{473}), and anti-phospho-Akt (Thr^{308}) from Cell Signaling Technology; and anti-phospho-Src (Tyr^{529}) from BioSource International.

Mice

EL mice are an inbred strain extensively studied as a model of human epilepsy or complex partial seizures (16). Convulsion can be induced in EL mice by physical stresses such as rhythmic vestibular stimulation. ASK mice were isolated from an EL colony as a strain that elicits no convulsion. Both EL and ASK mice were purchased from SEAC Yoshitomi and have been bred in the animal facility of the La Jolla Institute for Allergy and Immunology.

Cells and FcεRI stimulation

Bone marrow cells derived from EL and ASK mice (17) were cultured in IL-3-containing medium for 4–6 wk to generate >95% pure populations of mast cells (BMMC). Mast cells were sensitized overnight with 0.5 μg/ml anti-DNP IgE mAb, and stimulated with the indicated concentrations of Ag, DNP_{23}-human serum albumin (HSA) conjugates.

Proliferation in response to IL-3 and SCF

BMMC were incubated with the indicated concentrations of mouse rIL-3 or mouse recombinant SCF (both donated from Kirin Brewery) for 24 h. [^{3}H]Thymidine was added to the cultures for the last 8 h. Acid-insoluble tritium counts were measured in a scintillation counter.

Measurements of histamine, leukotrienes, and cytokines

Amounts of histamine in BMMC or in culture supernatants from BMMC that had been stimulated through the FcεRI with IgE and Ag were measured by HPLC as described (21). Leukotrienes C_4/D_4/E_4 accumulated in culture supernatants were measured by enzyme immunoassays (Amersham Biosciences). Supernatants of FcεRI-stimulated BMMC were also measured by ELISA for IL-2 and TNF-α (BD Pharmingen).

Immunoblotting analysis

Cells were lysed in 1% Nonidet P-40-containing lysis buffer (20 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 25 μM *p*-nitrophenyl *p*′-guanidinobenzoate, 1 μM pepstatin, and 0.1% sodium azide). In some experiments, this lysis buffer was supplemented with 1% *N*-dodecyl β-D-maltoside. Proteins in cleared cell lysates were either immunoprecipitated before, or directly analyzed by, SDS-PAGE followed by electroblotting onto polyvinylidene difluoride membranes (Millipore). Proteins reactive with primary Ab were visualized with an HRP-conjugated secondary Ab and ECL reagents (NEN Life Science Products).

In vitro kinase assays

Lyn, Fyn, c-Src, Syk, Btk, Cbp/PAG, and JNK1 molecules were immunoprecipitated from unstimulated or IgE/Ag-stimulated mast cells with appropriate Abs. Immune complexes were washed and incubated in the kinase buffer (50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 0.1 μM ATP) in the presence of 10 μCi [γ-^{32}P]ATP. In the case of Syk, immune complexes were incubated under the same conditions but with exogenous substrate, GST-HS1. For JNK assays, anti-JNK1 immunoprecipitates were incubated with GST-c-Jun (aa 1–79) at 30°C in 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 22 mM DTT, 20 mM β-glycerophosphate, 50 μM Na_3VO_4, 20 μM ATP, and 10 μCi [γ-^{32}P]ATP. Reaction products were analyzed by SDS-PAGE and blotted onto polyvinylidene difluoride membranes before exposure to x-ray films.

Transcriptional activity of the IL-2 gene reporter

EL and ASK BMMC (1×10^7 cells) were electroporated with 5–10 μg of IL-2Luc plasmid as described previously (22). Twenty-four hours after transfection, sensitization of the cells was initiated overnight with anti-DNP IgE. Forty-one hours after transfection, cells were left unstimulated or stimulated with 30 ng/ml DNP_{23}-HSA for 7 h before cell harvest. Cells were lysed in 0.2% Triton X-100 in 100 mM potassium phosphate (pH 7.8)/1 mM DTT. Luminescence of cleared cell lysates after the addition of ATP and luciferin solutions was measured using a Monolight 2010 luminometer (Analytical Luminescence Laboratory).

RT-PCR and Lyn cDNA cloning and sequencing

Total RNAs were isolated from EL and ASK mast cells using a TRIzol reagent (Invitrogen Life Technologies). DNAs complementary to these RNAs were synthesized using Reverse Transcriptase SuperScript II and oligo$(dT)_{12-18}$ (both from Invitrogen Life Technologies) as a primer. cDNAs were amplified in three segments that cover the entire coding regions of $p53^{lyn}$ and $p56^{lyn}$ by PCR using a high-fidelity *Pfu* DNA polymerase (Stratagene). Primers used for PCR will be provided upon request. Both strands of cDNAs were sequenced using a commercial sequencing kit (Applied Biosystems).

PCR primer pairs used for semiquantitative PCR were mouse Cbp/PAG (5′-CAGTAAGGTACGGACCTGCCT-3′ and 5′-AGCACACATGGGA GGTCTCC-3′) and mouse GAPDH (5′-ACCACAGTCCATGCCATC AC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′). PCR was performed under the following conditions: predenaturation at 94°C for 5 min, 35 cycles of DNA amplification (Cbp/PAG: 60 s at 94°C, 60 s at 57°C, 120 s at 72°C; GAPDH: 60 s at 94°C, 60 s at 59°C, 60 s at 72°C), and final elongation at 72°C for 7 min. Reaction products (248 bp for the Cbp/PAG band) were analyzed by electrophoresis on 1.5% agarose gels.

Results

Enhanced IL-3- and SCF-induced proliferation and increased FcεRI-stimulated cytokine secretion in ASK mast cells compared with EL mast cells

To understand the underlying mechanism for the difference between anaphylaxis-prone ASK mice and anaphylaxis-resistant EL mice, we investigated the phenotype of mast cells, the major effector cell type for anaphylaxis, in these mouse strains. Bone marrow cells were cultured in the presence of IL-3 to generate immature mast cells. The mice of both strains yielded morphologically pure (>95%) populations of mast cells after 4 wk of culture (data not shown). Mast cells from EL and ASK mice expressed similar levels of FcεRI and c-Kit on their cell surfaces, as measured by

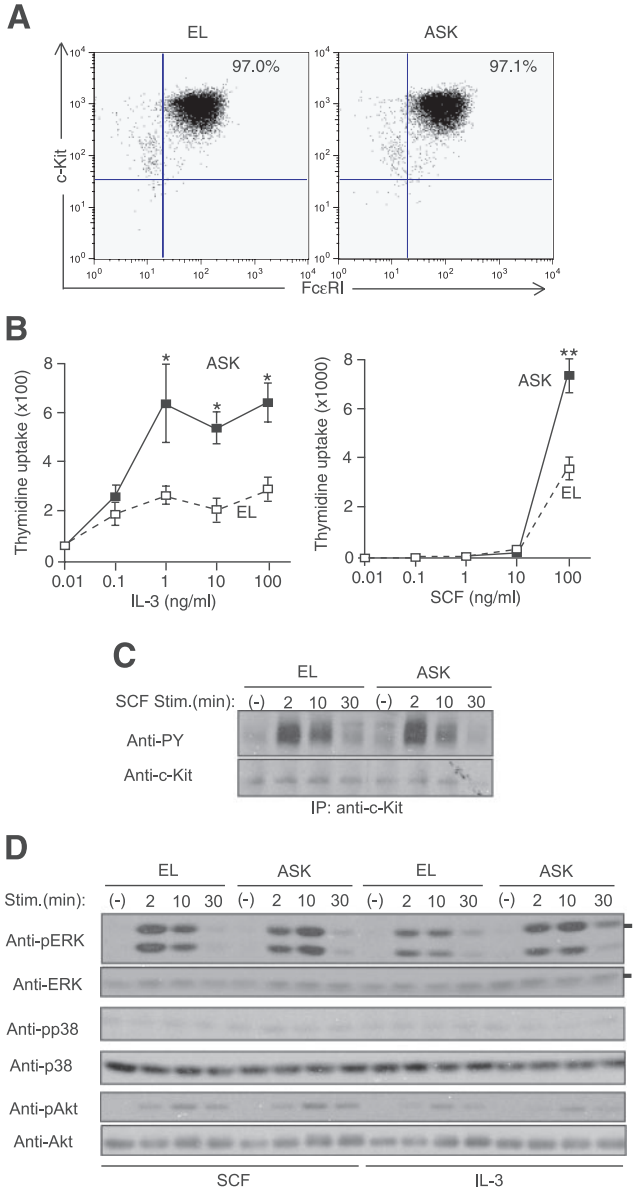

FIGURE 1. Expression of FcεRI and c-Kit in EL and ASK mast cells and increased proliferative responses to IL-3 and SCF in ASK mast cells. *A*, Surface expression of FcεRI and c-Kit was evaluated by flow cytometry. *B*, EL and ASK mast cells were incubated with the indicated concentrations of mouse rIL-3 or mouse recombinant SCF for 24 h. Thymidine uptake was measured during the last 8 h of culture. Representative results are shown of two similar experiments conducted. Errors represent SD. *, $p < 0.05$; **, $p < 0.01$ indicates statistical significance by Student's *t* test. *C*, EL and ASK mast cells were stimulated with 100 ng/ml SCF for the indicated periods. c-Kit was immunoprecipitated from cell lysates and immune complexes were analyzed by SDS-PAGE followed by immunoblotting. Blots were probed with anti-phosphotyrosine mAb, and then reprobed with anti-c-Kit. *D*, EL and ASK mast cells were stimulated with SCF or IL-3 (10 ng/ml). Cell lysates were analyzed by SDS-PAGE and immunoblotting with the indicated Abs. The positions of ERK1 are shown by a short dark bar (*right*).

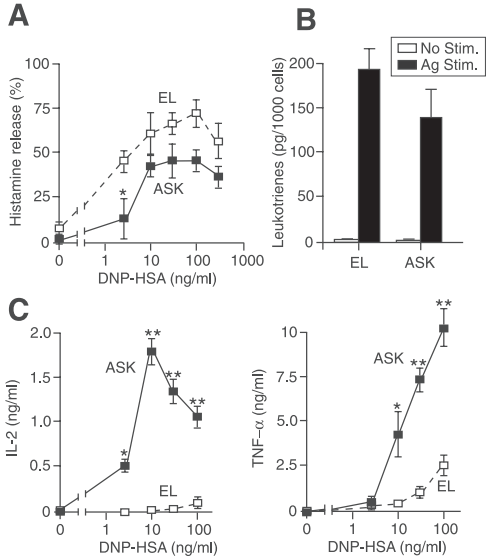

FIGURE 2. Decreased degranulation and increased cytokine production in FcεRI-stimulated ASK mast cells compared with EL cells. BMMC derived from EL and ASK mice were sensitized with anti-DNP IgE and stimulated with the indicated concentrations of DNP_{23}-HSA for 45 min for the measurement of histamine release (*A*), for 60 min for the measurement of leukotriene release (*B*), or for 20 h for the measurement of IL-2 and TNF-α secretion (*C*). Release of leukotrienes was induced by stimulation with 30 ng/ml DNP_{23}-HSA. Representative results are shown of three degranulation and four cytokine experiments conducted. Errors represent SD. *, $p < 0.05$; **, $p < 0.01$ indicate statistical significance by Student's *t* test.

flow cytometry (Fig. 1*A*). These data suggest that the genetic difference between these mice does not significantly affect the in vitro mast cell differentiation program. However, bone marrow cells from ASK mice in IL-3-containing medium yielded more BMMC than those from EL mice (data not shown). Consistent with this observation, proliferative responses to IL-3 and SCF were more vigorous in ASK than in EL cells (Fig. 1*B*). We examined signaling events in SCF- or IL-3-stimulated mast cells. Basal tyrosine phosphorylation of c-Kit was slightly higher in ASK than EL cells (Fig. 1*C*). However, SCF stimulation induced similar levels of c-Kit phosphorylation with similar kinetics in both mast cells. Among the tested downstream signaling molecules, we observed slower, but more prolonged phosphorylation of ERK1/2 and Akt in SCF-stimulated ASK cells (Fig. 1*D*). These results are consistent with stronger SCF-induced proliferation in ASK compared with EL cells. Upon IL-3 stimulation, ERK1/2 phosphorylation was higher in ASK cells than in EL cells. p38 phosphorylation was not induced by SCF or IL-3 stimulation.

When mast cells derived from EL mice were stimulated via the FcεRI with IgE and Ag, the cells responded by degranulation, leukotriene release, and cytokine production and secretion at levels similar to those from C57BL/6 or 129/SvJ mast cells. Mast cells derived from EL and ASK mice released histamine in an Ag dose-dependent manner, with ASK cells being slightly less efficient (Fig. 2*A*). Both cell types secreted similar levels of leukotrienes (Fig. 2*B*). These activation events are normally completed within 15 min. We next analyzed later activation events that take hours to reach completion. Strikingly, both IL-2 and TNF-α secretion 20 h after stimulation was at least 4- to 5-fold higher over Ag concentrations of 10–100 ng/ml in ASK mast cells compared with EL cells (Fig. 2*C*). The differences between EL and ASK mast cells in proliferative responses and cytokine production are similar to those between wild-type and $lyn^{-/-}$ mast cells (23–25).

Reduced Lyn kinase activity in ASK mast cells

To understand the molecular basis for the observed biological differences, particularly the dramatically enhanced cytokine secretion in ASK mast cells compared with EL mast cells, we first examined early signaling events. Ag stimulation of IgE-sensitized mast cells

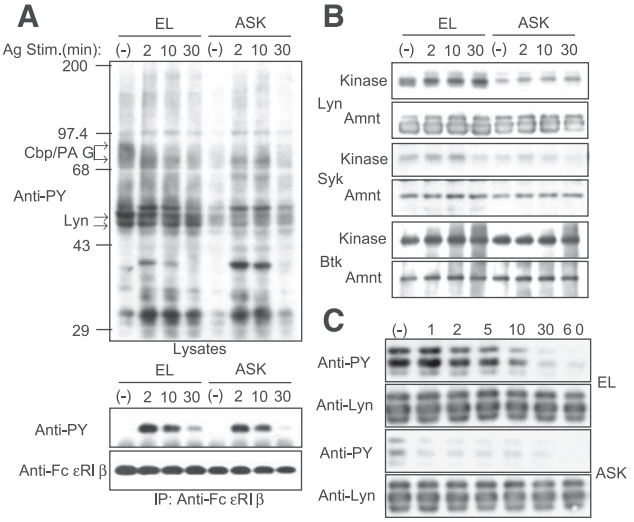

FIGURE 3. Reduced tyrosine phosphorylation and reduced catalytic activities of Lyn in ASK mast cells relative to EL cells. BMMC from EL and ASK mice were sensitized with anti-DNP IgE and stimulated with 100 ng/ml DNP_{23}-HSA for the indicated intervals. *A*, Tyrosine phosphorylation of proteins in the range of 28–200 kDa was evaluated by immunoblotting with anti-phosphotyrosine mAb. Positions of $p53^{lyn}$, $p56^{lyn}$, and Cbp/PAG are indicated. Tyrosine phosphorylation of FcεRI subunit β was examined by immunoprecipitation of FcεRI subunit β followed by immunoblotting with anti-phosphotyrosine (Anti-PY) mAb. Tyrosine phosphorylation of the 38-kDa band (the size of linker for activation of T cell) was apparently higher in ASK cells in this experiment. However, this result was not reproduced in other experiments. Linker for activation of T cell phosphorylation was similar between EL and ASK cells (data not shown). At least five phosphotyrosine blotting experiments were performed giving similar results. *B*, In vitro kinase assays were performed on Lyn, Syk, and Btk. Kinase products were analyzed by SDS-PAGE followed by blotting of the gels onto polyvinylidene difluoride membranes and finally by autoradiography of the blots. Autophosphorylated Lyn and Btk bands and GST-HS1 phosphorylated by Syk are shown (Kinase panels). The blots were reprobed with the respective precipitating Abs to show equal precipitation of the kinases (Amnt panels). Kinase results were reproduced at least one more time. Electrophoresis of Lyn kinase reactions were run longer than that of total cell lysates shown in *A*, which allowed the detection of the difference in mobility of Lyn bands between EL and ASK cells. *C*, Lyn was immunoprecipitated and immunoblotted with anti-phosphotyrosine (Anti-PY) mAb and then reprobed with anti-Lyn.

induced tyrosine phosphorylation of numerous cellular proteins in both strains. Interestingly, ASK cells had lower levels of tyrosine phosphorylation of 56- and 53-kDa proteins, which were among the most prominently tyrosine-phosphorylated proteins and corresponded to $p56^{lyn}$ and $p53^{lyn}$ proteins, respectively, in molecular mass, in resting as well as FcεRI-stimulated mast cells (Fig. 3*A*). By reprobing the same blot with anti-Lyn Ab, we confirmed that these bands comigrated with Lyn proteins (data not shown). Phosphorylation of a broad band of 75–90 kDa that included Cbp/PAG was also remarkably reduced in resting and FcεRI-stimulated ASK cells compared with EL cells (Fig. 3*A* and see below). However, tyrosine phosphorylation of other proteins was not generally lower in FcεRI-stimulated ASK mast cells. It is noteworthy that the tyrosine-phosphorylated protein band around 70–76 kDa containing Syk, Btk, HS-1, and SLP-76, was not strongly phosphorylated in EL or ASK cells, although it is one of the most heavily phosphorylated bands in BMMC derived from C57BL/6 and 129/SvJ mice, but not from CBA/J mice.

Next, we tested whether the apparently lower Lyn phosphorylation reflects the lower enzymatic activity of Lyn in ASK cells. In vitro autophosphorylation assays on anti-Lyn immunoprecipitates demonstrated that this effect is the case (Fig. 3*B*). Reprobing of the kinase blot and probing of the total cell lysates indicated that there is no significant difference in the amounts of Lyn proteins between EL and ASK cells (Fig. 3*B* and data not shown). Multiple experiments ($n = 6$) indicated that the enzymatic activity of Lyn in resting and FcεRI-stimulated ASK mast cells is substantially lower than activity in EL cells (relative Lyn kinase activity in EL vs ASK cells was 2.32 ± 0.12 at baseline and 3.83 ± 0.85 at 2 min of stimulation), although the possibility is not ruled out that this apparent difference in Lyn activity might be due to a difference in a Lyn-associated kinase between the two cell types. Tyrosine phosphorylation of immunoprecipitated Lyn proteins was higher in EL cells than ASK cells over the entire time course of up to 60 min of stimulation (Fig. 3*C*), supporting our interpretation that Lyn kinase activity is higher in EL cells than in ASK cells.

In a widely accepted current model, Lyn phosphorylates the ITAM of the signaling β and γ subunits of FcεRI followed by the recruitment of Lyn by phosphorylated β ITAM and Syk by phosphorylated γ ITAM (3). Therefore, we examined tyrosine phosphorylation of β and γ subunits. Despite the substantially lower Lyn activity, FcεRI-stimulated ASK cells exhibited only slightly lower levels of tyrosine phosphorylation of β and γ subunits than identically stimulated EL cells (Fig. 3*A*, *bottom*, and data not shown), suggesting that lower levels of Lyn kinase activity are sufficiently strong to phosphorylate these subunits or that other SFK compensate for the low Lyn kinase activity in ASK cells. Syk kinase activity was also lower in ASK cells than in EL cells (Fig. 3*B*), consistent with the previous study demonstrating that the full activation of Syk requires Lyn and Syk activity in mast cells (26). Catalytic activity of Btk is positively regulated by SFK and Syk (27, 28). Despite substantially lower activities of Lyn and Syk in ASK cells, Btk autophosphorylation activity was only slightly lower in ASK cells than in EL cells (Fig. 3*B*). Therefore, other SFK members might compensate for the lower Lyn kinase activity in Btk activation in ASK cells (see below).

Prolonged and/or increased activities of MAPK and Akt in ASK vs EL mast cells

Previous reports indicate that $lyn^{-/-}$ mast cells exhibit prolonged activation of MAPK, i.e., ERK1/2 and JNK1, increased Akt phosphorylation, and increased transcriptional activation of IL-2 promoter, culminating in the enhanced production and secretion of IL-2 and TNF-α (23, 29). Given the enhanced cytokine production and the lowered Lyn kinase activity in FcεRI-stimulated ASK mast cells, we examined other Lyn-regulated signaling events. As shown in Fig. 4*A*, both ERK1/2 and JNK1 were robustly activated in the both strains, but their activations were more sustained in ASK mast cells than in EL cells. ASK cells also exhibited higher levels of p38 MAPK phosphorylation. Akt activity, as measured by phosphorylation at Thr^{308} and Ser^{473}, was higher in ASK cells than in EL cells (Fig. 4*A* and data not shown). As expected from these results, transcriptional activity from the IL-2 promoter was much higher in ASK cells than in EL cells (Fig. 4*B*). Therefore, most, if not all, of the observed activation outcomes (degranulation and cytokine production), as well as the signaling events (activation of PTK, MAPK, and Akt and transcriptional activation), can be accounted for by the lower level of Lyn kinase activity in ASK vs EL mast cells.

Differential regulation of Lyn and other SFK in EL and ASK mast cells

Careful examination of the mobility of Lyn proteins on SDS-PAGE gels revealed a subtle difference between ASK and EL

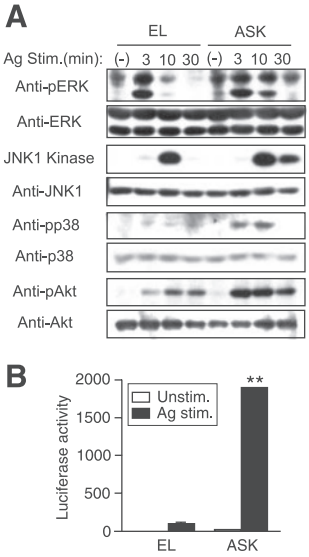

FIGURE 4. Prolonged or enhanced activation of MAPK, Akt, and IL-2 gene transcription in FcεRI-stimulated ASK mast cells compared with EL cells. *A*, Cells were stimulated as described in Fig. 2. Activities of ERK1 and ERK2, p38, and Akt were analyzed by immunoblotting of cell lysates with phospho-specific Abs that recognize activated states of these kinases. The blots were reprobed with relevant Abs that recognize the kinases irrespective of phosphorylation states. JNK1 activities were examined by in vitro kinase assays on JNK1 immunoprecipitates using GST-c-Jun (aa 1–79) as an exogenous substrate. The blot was reprobed with anti-JNK1 to show equal loading of JNK1 immunoprecipitates. *B*, EL and ASK mast cells were electroporated with the reporter plasmid IL-2Luc. IgE-sensitized cells were stimulated with 30 ng/ml DNP_{23}-HSA for 7 h before the measurement of luciferase activity. Errors represent SD. **, $p < 0.01$ indicate statistical significance by Student's *t* test.

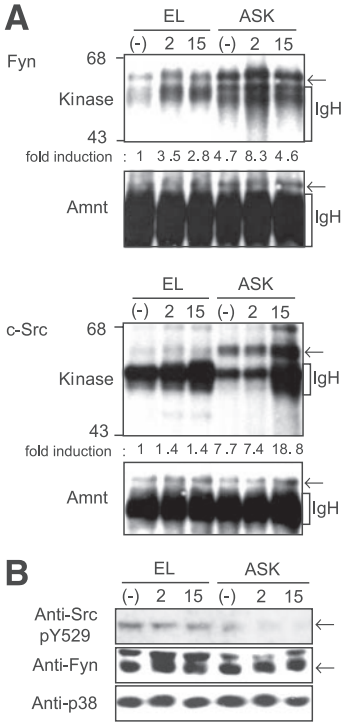

FIGURE 5. Increased autophosphorylating activities of Fyn and c-Src in ASK mast cells compared with EL cells. Cells were stimulated as described in Fig. 2. *A*, In vitro kinase assays were performed on immunoprecipitated Fyn and c-Src by autophosphorylation. Fyn and c-Src bands are indicated by the arrow and a broad band of H chains of Ig is also indicated. Fold induction of autophosphorylation estimated by densitometry is shown. Representative results are shown of three experiments conducted. *B*, Aliquots of the cell lysates were analyzed by immunoblotting with anti-Src phosphotyrosine (pY529), anti-Fyn, and anti-p38 Abs. The arrow indicates Fyn that exactly comigrated with the band detected by phospho-Src (pY529) Ab. The anti-p38 probing shows equal loading of lysates.

cells: Lyn proteins of both 56 and 53 kDa in ASK cells migrated slightly more slowly than those proteins in EL cells (Fig. 3*B* and this difference was observed in at least three more experiments). Differences in the mobility on an SDS gel could reflect differences in posttranslational modifications such as phosphorylation, or in the primary structure of the Lyn proteins. To test the latter possibility, we cloned three *lyn* cDNA clones each from mast cells of both ASK and EL mice by RT-PCR using a high-fidelity *Pfu* DNA polymerase and we determined their nucleotide sequences. No differences in the nucleotide sequence of the coding region of the *lyn* cDNAs were found between ASK and EL cells (data not shown), strongly suggesting that the reduced Lyn activity in ASK mast cells compared with that in EL mast cells is not due to a structural defect in Lyn, but due to an altered phosphorylation of Lyn kinase in ASK vs EL cells. Indeed, the difference in the mobility was eliminated after Lyn immunoprecipitates were treated with calf intestinal alkaline phosphatase and analyzed by SDS-PAGE (data not shown).

In addition to Lyn, mouse BMMC were reported to express several other SFK including Fyn and c-Src (5, 30). We next examined the enzymatic activity of Fyn and c-Src in in vitro kinase assays. Similar to $lyn^{-/-}$ cells (5, 24, 31), autophosphorylation activity of immunoprecipitated Fyn from resting and IgE/Ag-stimulated ASK mast cells were significantly higher than activity from EL cells (Fig. 5*A*), although comparable levels of Fyn protein were expressed in the two cell types (Fig. 5*B*). c-Src autophosphorylation activity was also higher in resting and IgE/Ag-stimulated ASK mast cells than in EL cells (Fig. 5*A*). Curiously, however, the ability of c-Src to phosphorylate the H chain of precipitating Abs was lower in ASK cells at early time points for an unknown reason, whereas Fyn activity in the same capacity was higher at all time points. Because enzymatic activity of SFK is negatively regulated by Csk-mediated phosphorylation at the C-terminal tyrosine residue (corresponding to Tyr^{529} in mammalian c-Src) (8), we analyzed the phosphorylation status of this residue of c-Src and Fyn by immunoblotting of mast cell lysates with Src Tyr^{529} phospho-specific Ab that recognizes the C-terminal tyrosine-phosphorylated c-Src and Fyn. Consistent with the kinase data, the negative regulatory tyrosine of c-Src/Fyn was hypophosphorylated in ASK cells compared with EL cells (Fig. 5*B*). Reprobing with anti-Fyn or anti-c-Src Ab indicated that this phosphorylated molecule is mainly Fyn, and the amounts of Fyn are comparable in EL and ASK cells. These results indicate that the catalytic activity of Lyn is regulated differently than that of Fyn and c-Src in EL vs ASK mast cells.

Reduced Cbp/PAG phosphorylation in ASK mast cells

It is proposed that the transmembrane adaptor protein Cbp/PAG, which resides in lipid rafts and binds Csk (9, 10), down-regulates the receptor-associated SFK activity by recruiting Csk to lipid rafts upon FcεRI aggregation (32). Therefore, we examined expression and phosphorylation levels of Cbp/PAG in EL and ASK mast cells. Immunoblotting analysis of cell lysates solubilized in 1% Nonidet P-40 revealed a drastic difference in Cbp/PAG: a fairly sharp protein band of 75 kDa was detected in ASK cells, whereas a broad band in the range of 70–90 kDa with a buried sharp 75-kDa band

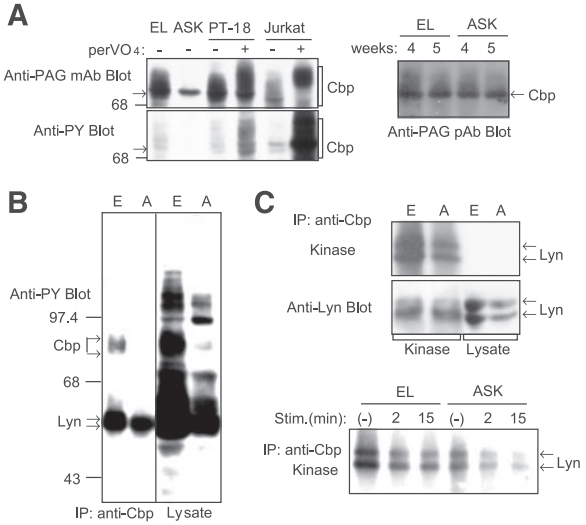

FIGURE 6. Interaction of Cbp/PAG with Lyn and reduced phosphorylation of Cbp/PAG in ASK mast cells. *A*, EL and ASK cells solubilized in 1% Nonidet P-40 were analyzed together with PT-18 mouse mast cells and Jurkat human T cells that were stimulated with (+) or not with (−) pervanadate for 5 min. Cbp/PAG was detected by immunoblotting with mAb PAG-C1 or anti-phosphotyrosine (Anti-PY) (*left*). EL and ASK BMMC generated by culturing bone marrow cells in IL-3 for 4 or 5 wk were analyzed by immunoblotting with anti-Cbp/PAG polyclonal Ab (*right*). Position of the sharp Cbp/PAG band of 75 kDa is shown by an arrow. *B*, Lysates of splenic cells from EL (E) and ASK (A) mice were either immunoprecipitated with anti-Cbp/PAG before, or directly subjected to, immunoblotting with anti-phosphotyrosine mAb. *C*, Cbp/PAG in unstimulated (*top panels*) or IgE/Ag-stimulated (*bottom*) EL and ASK mast cells was immunoprecipitated and immune complexes were subjected to in vitro kinase assays without exogenous substrate. Kinase products were analyzed by SDS-PAGE followed by blotting and autoradiography (*top panels*). The identity of 53- and 56-kDa bands as $p53^{lyn}$ and $p56^{lyn}$, respectively, was confirmed by reprobing with anti-Lyn Ab (*lower panel at top*).

within it was reproducibly detected in EL cells (Fig. 6*A*, *left*). Reprobing the same blot with anti-phosphotyrosine mAb showed the band patterns similar to those obtained by anti-PAG mAb, suggesting, but not proving, that the broad band is due to tyrosine phosphorylation (Fig. 6*A*, *left*). Similar band patterns were found (data not shown) when EL and ASK cells were lysed in 1% Nonidet P-40 with 1% *N*-dodecyl β-D-maltoside (a detergent that disrupts lipid rafts). The mAb PAG-01 used in the described immunoblotting experiments detects both phosphorylated and nonphosphorylated forms of Cbp/PAG. We next performed immunoblotting of 1% Nonidet P-40-lysed cells using a polyclonal anti-PAG Ab that detects preferentially nonphosphorylated forms of Cbp/PAG. Results indicated comparable expression of Cbp/PAG protein in EL and ASK BMMC that had been cultured in IL-3 for 4 and 5 wk (Fig. 6*A*, *right*). RT-PCR analysis demonstrated similar expression levels of Cbp/PAG mRNA in EL and ASK mast cells (data not shown). Therefore, these results suggest that Cbp/PAG is hypophosphorylated in ASK cells compared with EL cells. Reduced Cbp/PAG phosphorylation was also observed in spleen cells derived from ASK mice compared with EL mice (Fig. 6*B*). Alternatively, the streaky nature of the Cbp/PAG band might indicate ubiquitination.

Odom et al. (24) showed that Cbp/PAG phosphorylation is dependent mainly on Lyn in mast cells. Cbp/PAG is constitutively associated with Csk and recruits more Csk through the phosphotyrosine (of rat Cbp Tyr^{314}) Src homology 2 domain (of Csk) interaction upon FcεRI aggregation (32). Csk down-regulates the catalytic activity of SFK by phosphorylating the C-terminal negative regulatory tyrosine residue of the latter kinases (8). To identify which PTK is responsible for Cbp/PAG phosphorylation in EL and ASK mast cells, we first performed in vitro kinase assays using Cbp/PAG immune complexes precipitated with anti-Cbp/PAG. The results shown in Fig. 6*C* demonstrated the prominently phosphorylated 56- and 53-kDa proteins in both EL and ASK cells. Phosphorylation levels of these two proteins was lower in ASK than in EL cells, and FcεRI stimulation induced a reduction in intensity of the 56- and 53-kDa bands in both cells. Re-immunoprecipitation of Cbp/PAG-associated proteins with anti-Lyn confirmed that these proteins were in fact Lyn proteins. These results are consistent with those by Odom et al. (24). Therefore, we hypothesize that the reduced Lyn activity maintains low levels of Cbp/PAG phosphorylation in ASK cells, as well as low levels of Csk recruitment to lipid rafts. Reduced Csk recruitment in lipid rafts, in turn, can potentially explain higher catalytic activities of Fyn and c-Src in ASK cells.

Discussion

The present study identified a pair of mouse strains, EL and ASK, as exhibiting contrasting mast cell phenotypes similar to those of wild-type vs $lyn^{-/-}$ mice. Phenotypic similarities in mast cells between the EL and ASK mouse pair and the wild-type and $lyn^{-/-}$ mouse pair include higher proliferative responses to IL-3 and SCF and an excessive production of TNF-α and IL-2 in FcεRI-stimulated ASK and $lyn^{-/-}$ mast cells compared with their respective counterparts (Figs. 1 and 2). Mechanistically, these differences in FcεRI-induced phenotype can be accounted for by the lower Lyn activity in ASK cells, which is consistent with reduced Syk activity, prolonged ERK and JNK activation, increased p38 and Akt activation, and enhanced transcriptional activity of the IL-2 promoter. Both JNK and Akt were previously shown to be required for production of IL-2 and TNF-α (22, 33, 34). However, we also noticed differences in signaling between the EL and ASK and wild-type and $lyn^{-/-}$ pairs. The drastic reduction in FcεRI β subunit phosphorylation in $lyn^{-/-}$ cells was not seen in ASK cells. Linker for activation of T cell phosphorylation, which is dependent on Lyn, was similar in ASK and EL cells. Some of these disparities, and probably others, could be due to a reduction, but not abrogation, of Lyn activity in ASK cells unlike in $lyn^{-/-}$ cells.

At receptor-proximal levels, the reduced Lyn activity was reflected in the reduced Cbp/PAG phosphorylation and the increased catalytic activity of Fyn and c-Src in ASK mast cells. Together with analysis of $lyn^{-/-}$ cells, these data support the notion that Lyn phosphorylates Cbp/PAG within lipid rafts (both Lyn and Cbp/PAG localized to lipid rafts). Therefore, the negative regulatory feedback mechanism (Lyn activation→Cbp/PAG phosphorylation→Csk recruitment→inhibition of SFK) does not function efficiently in ASK cells compared with EL cells. Dependency of Cbp/PAG phosphorylation on Lyn may be mast cell-specific. Indeed, Fyn was shown to associate with Cbp/PAG in human T lymphocytes (10), but not in rat brain (9), and Fyn in lipid rafts plays an essential role in Cbp/PAG phosphorylation in T cells (35). These data suggest that SFK dependency of Cbp/PAG phosphorylation may hinge on relative amounts of individual SFK members available in the proximity of Cbp/PAG in lipid rafts. This argument is consistent with the well-known facts that Lyn is the most predominantly expressed SFK in mast cells and mostly associated with lipid rafts (36) and that Cbp/PAG phosphorylation is not reduced in $fyn^{-/-}$ cells (24). It is also noteworthy that, similar to Lyn, a high proportion of Fyn is localized to lipid rafts, but expression levels of Fyn appear to be lower than Lyn in mast cells. Lipid raft localization requires Lyn to be both myristylated and palmitylated at its N-terminal region, whereas c-Src, lacking a palmitylation

site, cannot be localized to lipid rafts. Therefore, slight differences in the regulation of Fyn vs c-Src in EL and ASK cells could be accounted for by the difference in localization to lipid raft (Fyn) vs nonlipid raft (c-Src) compartments. Another possibility, which is not mutually exclusive with the above-mentioned scenario, is that individual SFK members may have distinctively different relationships with Csk and/or other regulatory proteins (e.g., phosphatases). These arguments might well explain the preferential use of Fyn in T cells and Lyn in mast cells in the phosphorylation of Cbp/PAG. Potentially confounding regarding the presumed role of Cbp/PAG in regulation of SFK, mice deficient in Cbp/PAG were recently shown to have no defects in thymic development or T cell functions; moreover, recruitment of Csk to lipid rafts was not impaired substantially (37) or not at all (38). Therefore, the role of Cbp/PAG in mast cells should be directly studied with the mutant mice.

The exact nature of the initial event within the EL mouse colony leading to the isolation of epilepsy-resistant and anaphylaxis-prone ASK mice is unknown. The primary event could have been a mutation in a molecule that regulates the Lyn kinase activity. However, there are numerous candidate molecules that can regulate Lyn and other SFK. We observed comparable levels of surface expression of CD45, a protein tyrosine phosphatase that can dephosphorylate SFK, in EL and ASK mast cells, but this interesting pair of mice warrants further examination of this and other candidate molecules that definitively distinguish their phenotypes.

Unlike EL mice, ASK mice are susceptible to IgE-dependent anaphylactic stimuli (17, 18). Is this trait of ASK mice dependent on the mast cell phenotype featured by the reduced Lyn activity? This possibility is likely, although cells (e.g., endothelial cells) involved in anaphylaxis other than mast cells have not been studied in these mice. ASK mast cells exhibit increased cytokine production and secretion in response to FcεRI aggregation. Moreover, the increased TNF-α secretion may contribute to an increased anaphylactic response (39). Indeed, young $lyn^{-/-}$ mice exhibit increased susceptibility to anaphylactic stimuli (24) and have higher IgE levels (40). As $lyn^{-/-}$ mice age, their serum IgE levels increase as well as occupancy of FcεRI with IgE. Unlike the increased level in mast cell activation that is dependent on Fyn, the increased IgE is not the consequence of increased Fyn kinase (24). In contrast, mice with a gain-of function *lyn* mutation have reduced serum IgE levels (41).

Differential regulation of SFK can be involved in epileptogenesis. SFK are expressed in the CNS and have been implicated in the regulation of glutamate *N*-methyl-D-aspartate (NMDA) receptors, affecting neural excitability and plasticity (42–44). Phosphorylation of NMDA receptors by Fyn and Src has been shown to increase NMDA receptor channel activity (42, 45) and mediate the induction of long-term potentiation in the CA1 region of the hippocampus. Enhanced NMDA responses were observed in the kindling model of epilepsy (46–49) and human epilepsy (50). Observations from experiments using Fyn-deficient and Fyn-transgenic mice and pharmacological experiments support a role for SFK in the induction of epilepsy (51–54). There may be a mechanistic relation involving SFK between the epileptogenic trait and the anaphylactogenic trait in EL and ASK mice. In this regard, it will be interesting to test whether Lyn-deficient mice or other SFK-deficient mice exhibit reduced susceptibility to epileptogenic stimuli.

In summary, our analysis of mast cells derived from anaphylaxis-prone ASK and anaphylaxis-resistant EL mice demonstrated the differential regulation of SFK as the basis of differences in mast cell activation between these mice, once again confirming the crucial importance of this class of early activating and inhibitory signaling PTK in FcεRI-mediated mast cell activation. Differences in Cbp/PAG phosphorylation may be an underlying mechanism for the differential regulation of catalytic activity of Lyn and other SFK. Further study of these mice will likely provide insight into the mechanisms of SFK regulation as well as pathogenetic processes of anaphylaxis and epilepsy.

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Disclosures

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