

The Limited Contribution of Fyn and Gab2 to the High Affinity IgE Receptor Signaling in Mast Cells

Running Title: Syk, Fyn and Gab2 in mast cell signaling

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Several studies with mast cells from knockout mice have suggested that the tyrosine kinase Fyn and its downstream substrate Gab2 may play a role in high affinity IgE receptor (FcεRI) mediated mast cell activation. To better understand the role of these two molecules and of Syk, we transiently transfected mast cells with small interference RNA (siRNA) targeted to Fyn, Gab2 or Syk to specifically decrease their expression. The siRNA suppression of Gab2 but not Fyn reduced activation of the phosphoinositide-3-kinase (PI3K) pathway as demonstrated by the change in phosphorylation of Akt; this indicates that Gab2 but not Fyn regulates this pathway. The decreased expression of Gab2 and Fyn had minor effects on degranulation. There were also some minor changes in activation of the NFAT or NFκB transcription factors in cells with reduced expression of Fyn or Gab2. Decreased Gab2 but not Fyn reduced the FcεRI-induced activation of the Erk, Jnk and p38 MAP kinases and the release of TNF-α. In contrast, decreased expression of Syk dramatically reduced FcεRI-induced degranulation, activation of NFAT and NFκB. Therefore the reduction in expression of these proteins in mast cells suggest that Syk is the major regulator of FcεRI-mediated reactions while Fyn has minor if any effects and Gab2 regulates primarily late events including MAP kinase activation and release of cytokines.

INTRODUCTION

The clustering of the high affinity receptor for IgE (FcεRI) by multivalent antigen on mast cells results in degranulation, with the release of inflammatory mediators such as leukotrienes and cytokines (1-3). In this signaling pathway phosphorylation of the immunoreceptor tyrosine-based activation motif of the β and γ subunits of FcεRI recruits and activates Syk which is critical for the downstream propagation of signals (4-9). Syk-mediated phosphorylation and/or activation of phospholipase Cγ, LAT, SLP-76 and PI3K are important for calcium mobilization and degranulation (7,10-12). The increase in intracellular calcium concentration leads to activation of molecules including MAPKs Erk, p38 and JNK, the transcription factors NFκB and NFAT (13-19), all of which play important roles in the control of IgE-mediated cytokine synthesis.

After FcεRI aggregation the Src family kinase Lyn is thought to be the enzyme that phosphorylates the tyrosine residues of the immunoreceptor tyrosine-based activation motif of the β and γ subunits of the receptor (3,20,21). However, bone marrow-derived mast cells (BMMC) from Lyn^{-/-} mice have normal or enhanced FcεRI-induced degranulation, suggesting that other tyrosine kinases could substitute for Lyn (22-26). Such a kinase could be Fyn, which is activated following IgE-FcεRI stimulation and its tyrosine phosphorylation is increased in Lyn negative cells (22,27). In BMMC from Fyn deficient mice, there is decreased PI3K activation as reflected by Akt tyrosine

phosphorylation, with reduced activation of JNK, p38 MAPK, and of the transcription factor NF κ B; these changes parallel decreased degranulation and release of leukotrienes (27,28).

Studies of Fyn deficient BMMC suggest an interaction between Fyn and the adaptor protein Gab2 (27). Gab2 is tyrosine phosphorylated after Fc ϵ RI stimulation and has potential binding sites for signaling molecules such as Grb2, SHP2, p85 subunit of PI3K and Shc (27,29,30). Mast cells from Gab2^{-/-} mice show decreased Kit-mediated signal transduction resulting in decreased proliferation *in vitro* and lower number of mast cells in various tissues *in vivo* (31,32). Bone-marrow derived mast cells from Gab2^{-/-} mice have decreased activation of PI3K, reduced tyrosine phosphorylation of phospholipase C γ 1, Akt and JNK resulting in reduced Fc ϵ RI-induced degranulation and cytokine generation (33). In Fyn deficient cells there is decreased tyrosine phosphorylation of Gab2 while in Syk^{-/-} cells there is complete absence of Gab2 phosphorylation (27,32). Gab2 might regulate mast cells degranulation by interfering with the granule translocation to the plasma membrane, through a calcium-independent mechanism that also involves Fyn and the small GTPase RhoA (34). These studies with Fyn and Gab2 deficient bone marrow mast cells suggest a role for these proteins in signaling from Fc ϵ RI.

In the present experiments, we used transient transfection with small interference RNA (siRNA) targeting Syk, Fyn or Gab2 to investigate the relative roles of these molecules in signaling in mast cells. The changes in cellular functions were monitored for a short period to limit the compensatory changes in cells that may occur due to gene inactivation. Treatment with siRNA specifically decreased by >75% the expression at the protein level of Syk, Fyn and Gab2 at 48-72h after transfection; Syk knockdown significantly reduced IgE-Fc ϵ RI-mediated degranulation, and NF κ B or NFAT activation. However, the decreased expression of Fyn or Gab2 had minor if any effects on degranulation, and NFAT or NF κ B activation. The siRNA suppression of Gab2 but not Fyn decreased PI3K activation as indicated by changes in phosphorylation of Akt, suggesting that Gab2 but not Fyn regulated PI3K. The decreased expression of Gab2 also resulted in reduced

phosphorylation of the MAPKs p38 and JNK, as well as decreased TNF- α release. Taken together these findings suggest that Fyn does not appear to play a major role in IgE-Fc ϵ RI-mediated mast cell responses whereas Gab2 has a role in generation of cytokines.

EXPERIMENTAL PROCEDURES

Materials and Antibodies- Mouse IL-3 and stem cell factor (SCF) were purchased from Invitrogen (Carlsbad, CA). The anti-JNK antibody (Abs) were from Upstate Biotechnology (Lake Placid, NY); anti-Syk (N-19), anti-Fyn (H-80), anti-Gab2 (M-19), anti-phospho-JNK Abs were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-Gab2 Tyr452, anti-phospho-Gab2 Ser159, anti-phospho-Akt Ser473, anti-Akt, anti-phospho-p38, anti-p38, anti-phospho-p44/42 MAPK, and anti-p44/42 MAPK Abs were from Cell Signaling Technology (Beverly, MA). All other materials were used as previously described (30).

Cell Culture- The mouse mast cell line MMC-1 (CXBI-I-CA5) was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% heat-inactivated FBS, 2 mM L-glutamine, 5 X 10⁻⁵ M β -mercaptoethanol, 10 % NCTC 109 media, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and antibiotics as described previously (35). The growth factor dependent mouse mast cell line MC9 was cultured in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 6 mM L-glutamine, antibiotics, 1.1 X 10⁻⁴ M 2- β -mercaptoethanol, 10 ng/ml IL-3, and 10 ng/ml SCF. To maintain viability all cell lines were subcultured every 2 to 3 days.

To generate the stable Fc ϵ RI-controlled NFAT reporter cell line, MC9 cells were transfected with a plasmid that had three tandem NFAT-binding sites fused to eGFP under the control of an minimal IL-2 promoter (36). Transfected cells were stimulated with IgE plus antigen, and GFP positive cells were collected by sorting using a FACStar Plus (Becton Dickinson). After several cycles of stimulation and sorting, GFP positive single-cell clones were selected for expansion that

showed 20- to 30-fold increased GFP expression on IgE-antigen stimulation.

To generate the stable NF κ B reporter cell line, MC9 cells were transfected with a transcriptional reporter plasmid carrying four copies of the consensus NF κ B binding site upstream of the minimal cytomegalovirus promoter that regulated NF κ B-dependent GFP expression (System Biosciences, Mountain View, CA, USA). Selection of a cloned cell line was as described for the NFAT reporter cell line.

Bone marrow cells were collected from healthy 2-4 months old C57Bl/6 females and cultured for up to 6 weeks in DMEM supplemented with 20% heat-inactivated FBS, 2 mM L-glutamine, 5×10^{-5} M β -mercaptoethanol, 10 % NCTC 109 medium, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, antibiotics, 30 ng/ml IL-3 and 25 ng/ml SCF. Bone marrow derived mast cells (BMMC) were used for experiments starting with the 4th week of culture.

Transient Transfection with siRNA - siGENOME ON-TARGETplus SMARTpool targeting Syk, Fyn and Gab2, each containing four siRNA duplexes per gene, were purchased from Dharmacon (Lafayette, CO). Catalogue numbers and sequences are specified in the supplemental information (Supplemental Table 1). For control, a pool containing four siRNAs with minimal effect on known mouse genes (ON-TARGET plus siCONTROL non-targeting pool, cat#: D-001810-10-20) was also from Dharmacon. Transfection was by using Amaxa Nucleofector 96-well Shuttle System. For transfection, cells were centrifuged and washed once with DMEM without FCS and then re-suspended in Amaxa's cell line 96-well nucleofection solution SF and distributed into wells at 0.5×10^6 cells per transfection (MC9 NFAT-GFP and MMC-1) or 0.7×10^6 cells per transfection (MC9 NF κ B-GFP and BMBCs). The siRNA for Syk, Fyn or Gab2 and for control, non-targeting siRNA were added at 0.25 or 0.5 μ g/ 10^6 cells. The programs used for transfection were DS-137 (for MC9 cells), FF-138 (for MMC-1), and DC-100 (for BMBCs). After shocking the cells, 95 μ l of calcium free medium warmed at 37°C was added immediately into each well (Minimum Essential Medium Eagle without calcium supplemented with 2 mM L-glutamine, 5×10^{-5} M β -mercaptoethanol, 10% NCTC 109 media, 0.1 mM

non-essential amino acids, 1 mM sodium pyruvate, and antibiotics). After 10 min at 37°C, the cell suspension was mixed, and transferred into 96 well plates containing culture medium. Under these conditions an enhanced green fluorescent protein-expression vector resulted in more than 80% of the cells becoming fluorescent after 24 hours of transfection. Knock down in the expression of Syk, Fyn and Gab2 was determined by immunoblotting the cell pellets after transfection.

Degranulation Measurements- The β -hexosaminidase release was determined in transfected MMC-1 and BMMC cells that were sensitized for 24h with 0.3 μ g/ml antigen-specific IgE (anti-TNP mAb TIB-142). Aliquots of cells from each transfection were used in duplicate to determine spontaneous or Fc ϵ RI-induced degranulation by incubating for 45 min with DMEM-TB (DMEM containing 10 mM Tris, pH 7.5 and 0.1% BSA plus SCF for BMMC) or DNP₃₅-BSA antigen in DMEM-TB. The cell pellets from wells incubated only with DMEM-TB were solubilized with 1% Triton X-100 in DMEM-TB (blanks), and used to determine the total cellular content of β -hexosaminidase while pellets from antigen-stimulated cells were used for immunoblotting as described below. The enzymatic activity of β -hexosaminidase was measured with *p*-nitrophenyl *N*-acetyl-D-glucosaminide in 0.1 M sodium citrate (pH 4.5) for 60 min at 37°C. The reaction was stopped by the addition of 0.2 M glycine pH 10.7. The release of the yellow product 4-*p*-nitrophenol was detected by absorbance at 405 nm. Degranulation of cells treated with siRNA for Syk, Fyn or Gab2 is presented as percent of the release determined for cells transfected with the control siRNA.

Cytokine Release Measurements- Quantitative measurements of TNF- α , IL-13 and CCL-4 (MIP-1 β) release from MC9, MMC-1 and BMBCs were performed with cytokine specific Quantikine ELISA Kits (R&D) according to the manufacturer's instructions.

Flow Cytometric Measurements of NF κ B and NFAT Activation- Fluorescence of the GFP reporter was used as a marker of Fc ϵ RI-induced NF κ B or NFAT activation after transfection with siRNA for Syk, Fyn, Gab2 or siCONTROL. Cells were distributed into 96-plates containing supplemented DMEM, primed for 24h with 0.3

μg/ml of IgE and then stimulated by antigen for 5h (for NFκB activity) or 18h (for NFAT activity). The NFκB activity was measured 24h, 48h, and 72h post-transfection. NFAT-induced expression was measured at 48h, 72h and 96h post-transfection because the maximum GFP expression in these cells required longer culture. The Mean Fluorescence Intensity (MFI) of treated and control cells was determined by a FACScan 3 color analysis cytometer and processed by CellQuest software (BD Bioscience, San Jose, CA). The activation in the treated cells is presented as percentage of that determined for the control cells. A typical flow cytometry analysis (FACS) of GFP expression in NFAT expressing reporter cells treated with siRNA specific for Syk is presented in Supplemental Fig. 1.

Cells Activation and Immunoblotting- Cell pellets from all transfections with siCONTROL or siRNA for Syk, Fyn and Gab2 were solubilized and the lysates analyzed by immunoblotting with the corresponding antibody and anti-FcεRIβ. In experiments to study MAP kinase activation, aliquots of IgE-sensitized cells were incubated for 2, 5 and 10 min with medium or the optimal antigen concentration. To study phosphorylation of Akt, transfected cells were cultured for 24h, then sensitized with for 18h either in SCF-free DMEM (MC9 cells) or FCS-free DMEM containing 1% BSA (MMC-1 cells). Each transfected sample was then divided into six aliquots; two of these were used to assess cellular responses under starvation conditions (activation of the transcription factors or degranulation, as described above), the remaining four aliquots were used for control, or 2, 5 and 10 min of antigen stimulation. Activation was stopped by adding 5 volumes of ice-cold PBS containing 5 mM EDTA and 2 mM Na₃VO₄. Samples were briefly centrifuged at 8,000 rpm, the supernatants removed, and pellets were immediately boiled for 10 min in 2x SDS-sample buffer containing 2% β-mercaptoethanol. Proteins were resolved on precast minigels (Novex Experimental Technologies, San Diego, CA), and electrophoretically transferred onto PVDF Immobilon filters (Millipore, Bedford, MA). After blocking nonspecific reactivity with 3% BSA, filters were probed with specific Abs and proteins were detected using an enhanced chemiluminescence detection kit (Perkin Elmer, Boston, MA).

Densitometry- The blots were scanned and processed on KODAK Image Station 4000MM Digital Imaging System (Carestream Molecular Imaging, Rochester, NY). The densitometry of the bands was used to calculate the decrease in the protein expression as a percentage of control cells transfected with non-targeting siRNA using the values of the anti-FcεRIβ blot to correct for gel loading.

Data Analysis- Paired two-sided Student t-tests were used to determine significance; the specific p values are indicated in the figures and are as follows: (*) for $p \leq 0.05$; (**) for $p \leq 0.01$; and (***) for $p \leq 0.001$. Number of experimental repeats (n) is specified in each figure legend.

RESULTS

Decreased Expression of Syk but not Fyn or Gab2 Reduces FcεRI-induced Degranulation. Transient transfection with siRNA targeting Syk, Fyn or Gab2 was used to investigate the role of these molecules in mast cell signaling. MMC-1 cells were efficiently transfected with siRNA and maintained their degranulation response after transfection. In all experiments, the expression of proteins was monitored by immunoblotting. Because the variability in half-life of different proteins causes different lag times between siRNA transfection and the maximum decrease in protein expression, the measurement of β-hexosaminidase release was performed at 24, 48, and 72 hours post transfection.

Studies with cells lines and bone marrow derived mast cells have shown that Syk is essential for FcεRI-induced degranulation (7,8). Similarly, the siRNA-induced reduction in expression of Syk correlated with a decrease in the cellular response: a 41%±14% (n=6) decrease of Syk expression after 24h of siRNA treatment caused a 36%±13% reduction of degranulation; a 76%±9% decrease in Syk at 72h caused a corresponding 72%±9% reduction in degranulation (Fig. 1). The tyrosine kinase Fyn and the adaptor Gab2 have also been reported to contribute to IgE-FcεRI-mediated degranulation in mast cells (27,33). We therefore investigated whether the siRNA-induced decrease in expression of Fyn and Gab2 correlated with the changes in degranulation. Treatment with siRNA effectively reduced the expression of Fyn or Gab2

as shown by a representative immunoblot (Fig. 1A) and the densitometry data of all experiments (Fig. 1B). The maximum decrease in Fyn expression was at 48h and 72h after transfection ($79\% \pm 10\%$), while the Gab2 levels were the lowest after 48h ($82\% \pm 6\%$). However, IgE-Fc ϵ RI-mediated degranulation with the optimal antigen concentration did not significantly change in these cells, regardless of the dramatic decrease in expression of Fyn or Gab2 (Fig. 1C).

We also investigated the response under wider stimulation conditions 48h after transfection, when expression of Gab2 and Fyn were at the lowest levels (Fig. 1D). Syk-specific siRNA treatment caused decreased degranulation with all tested antigen concentrations, except with 100 ng/ml. In cells treated with siRNA for Fyn or Gab2 there were minor changes in degranulation; with Gab2 there was slight enhancement that was statistically significant while with Fyn the reduction at low antigen concentrations and increase at higher levels was not statistically significant. These findings confirm the critical role of Syk in IgE-Fc ϵ RI-mediated degranulation and suggest a possible role for Gab2 as a negative regulator, and indicate minimal involvement of Fyn.

Decreased Expression of Fyn or Gab2 has Minor Effects on Fc ϵ RI-induced Activation of NF κ B and NFAT. The expression of new genes and cytokines after Fc ϵ RI stimulation depends on signals that are generated and transmitted to the nucleus. These signals include the two transcription factors, NFAT and NF κ B. MC9 reporter cells in which NFAT or NF κ B regulate expression of GFP were used to determine the changes in these transcription factors in cells with reduced expression of Syk, Fyn or Gab2. In the NF κ B reporter cells, Fc ϵ RI-induced GFP expression was maximal after 5 hours, while in the NFAT cells it was 18 hours after receptor stimulation (data not shown). Therefore, the antigen-induced activation of these transcription factors in siRNA-treated cells was determined after 24h to 72h post-transfection for NF κ B, and after 48h to 96h post-transfection for NFAT. After FACS analysis, the remaining cells were analyzed by immunoblotting to confirm the knockdown of the target proteins (Fig. 2, A and C). Decreased expression of Syk reduced the activation of both NF κ B and NFAT (Fig. 2, B and D); there was

decreased NF κ B-induced GFP expression following 24 to 72 hours of siRNA treatment with the maximal reduction in NF κ B activity ($50\% \pm 8\%$) after 48h (Fig. 2B). In NFAT-GFP cells the siRNA-induced decrease in Syk expression was maximal after 72 to 96 hours with a parallel decrease in NFAT activation (Fig. 2D).

As with the other cell line, there was efficient decrease in expression of Fyn with the siRNA in these MC9 NF κ B-GFP and NFAT-GFP reporter cells; the most decrease was at 48 hours after transfection ($71\% \pm 9\%$ to $81\% \pm 6\%$) (Fig. 2, A and C; densitometry analysis of all experiments in Supplemental Fig. 2). This Fyn knockdown caused minimal changes in the Fc ϵ RI-induced activation of both NF κ B and NFAT. Thus, there was some increase in NF κ B activation at 24-48 hours ($15\% \pm 6\%$ at 48h of treatment), which appeared to parallel the changes in expression of Fyn (Fig. 2B and Supplemental Fig. 2A). However, the changes in NF κ B-induced GFP expression were only significant at the 24h determination. NFAT activation (Fig. 2D) was reduced ($21\% \pm 8\%$) at 48 to 96 hours with only the 96h values being statistically significant, although the decrease in Fyn protein expression was maximal at earlier time points (Fig. 2C; Supplemental Fig. 2B). Gab2 knockdown was more transient though there were still efficient reduction in protein expression at 48 hours in both reporter cell lines; $76\% \pm 9\%$ in NF κ B-GFP and $75\% \pm 10\%$ in NFAT-GFP cells (Fig. 2, A and C and Supplemental Fig. 2). The decreased Gab2 expression slightly reduced NF κ B activation ($21\% \pm 9\%$) at 24h (Fig. 2B) but had no effect on NFAT activation at any of the time points (Fig. 2D). These findings confirm the central role of Syk and suggest a small role for Fyn and Gab2 in Fc ϵ RI-mediated late signaling events in mast cells.

Gab2 but not Fyn Regulated IgE-dependent Akt Phosphorylation. In mast cells, IgE-Fc ϵ RI-induced Akt activation is regulated by Syk and PI3K and thus can be used as a measure of PI3K activation (7-9). Akt by controlling NF κ B and NFAT is implicated in cytokine synthesis (16). There is defective Fc ϵ RI-dependent PI3K signaling in Gab2^{-/-} BMMC (33) while cells from Fyn^{-/-} mice had impaired phosphorylation of Gab2, PI3K and Akt (27). We therefore examined Akt phosphorylation to determine if there were

changes in the PI3K pathway after reduced expression of these proteins. Phosphorylation of Akt was evaluated 48h post-transfection as this length of siRNA treatment reduced the expression of Syk by at least 50% and that of Fyn and Gab2 by >75%. In the MC9 NFAT or NF κ B reporter cells treated with Syk siRNA there was reduced Fc ϵ RI-induced Akt phosphorylation; the greatest decrease (52% $\pm$ 11%) was at 10 min after antigen stimulation (Fig. 3, A and B). Fyn specific siRNA treatment did not change the extent or time course of Akt phosphorylation while the decreased expression of Gab2 reduced Akt phosphorylation (Fig. 3B). There was a similar pattern and extent of the siRNA-induced changes in phosphorylation of Akt in the MMC-1 cells used for the degranulation experiments (Supplemental Fig. 3, A and B). These results indicate that Fc ϵ RI-induced activation of PI3K-Akt was downstream of Syk or Gab2, but that it was not regulated by the decreased expression of Fyn.

The Fc ϵ RI induced phosphorylation of Gab2 is reduced in BMMC from Fyn^{-/-} mice (27,32). We therefore tested cell lysates from Fyn-specific siRNA treated cells for Gab2 phosphorylation at two specific sites (Ser159 and Tyr452) that have been identified as important for signaling by this protein. In fibroblasts, Akt phosphorylates Ser159 of Gab2, a site that is a negative regulator of Gab2 function (37) while results in yeast two-hybrid assays suggest that Tyr452 on Gab2 recruits p85, the regulatory subunit of PI3K (38). The decreased expression of Fyn did not change the pattern or extent of phosphorylation of Gab2 Ser169 or Tyr452 after IgE-antigen stimulation (Fig. 3C; Supplemental Figure 3C). Thus, Fc ϵ RI-activated phosphorylation of Gab2 at these two sites does not depend on Fyn.

Gab2 but not Fyn Regulates Fc ϵ RI-mediated MAPK Phosphorylation. MAPKs are involved in the Fc ϵ RI-induced signaling that results in the release of arachidonic acid-derived products and cytokines (39,40). BMMC from Fyn^{-/-} or Gab2^{-/-} mice have defects in MAPK activation (27,33). We therefore investigated whether the short term decreased expression of Fyn or Gab2 had effects on the MAPK pathways. There was phosphorylation of Erk, p38 and JNK after 2, 5 and 10 min of Fc ϵ RI stimulation of control MC9 cells (Fig. 4). However, there were no significant

changes in this phosphorylation pattern in cells with decreased expression of Fyn. In contrast, Gab2-specific siRNA treatment resulted in decrease in the antigen-induced phosphorylation of these MAPKs. The largest decrease was at 5 and 10 min after antigen stimulation for phosphorylation of p38 (45% $\pm$ 8%) (Fig. 4, C and D) and JNK (72% $\pm$ 4%) (Fig. 4, E and F), while Erk phosphorylation was decreased at 5 min of antigen exposure (61% $\pm$ 13%) (Fig. 4, A and B). There were similar changes in antigen-induced phosphorylation patterns of Erk, p38 and JNK in siRNA-treated MMC-1 cells (Supplemental Fig. 4). These results suggest that decreased Gab2, but not Fyn expression, reduced the Fc ϵ RI-induced activation of the Erk, p38 and JNK MAPKs.

Fc ϵ RI-mediated TNF- α Secretion was Decreased by Reduced Expression of Gab2 but not of Fyn. The above results indicated that Fyn and Gab2 have different effects on late Fc ϵ RI signaling events in mast cells. Antigen-induced TNF α secretion was used to test whether these signaling alterations were accompanied with changes in the secretion of newly synthesized mediators. Release of TNF α was measured in MC9 and MMC-1 cells treated with siRNA for 48 hours. As in previous experiments, there was dramatic decrease in the expression of Fyn and Gab2 in these treated cells (Fig. 5, B and D). The reduced expression of Fyn did not result in any significant change in TNF α release. In contrast, with Gab2 there was significant inhibition of TNF α release in both MMC-1 (32% $\pm$ 8%; Fig. 5A) and MMC-9 (55% $\pm$ 3%; Fig. 5C). These results, together with changes in phosphorylation of Akt and the MAPKs, suggest that the reduced expression of Gab2 but not Fyn regulates late events in Fc ϵ RI-stimulated mast cells.

Decreased Expression of Gab2 but not of Fyn Reduced Fc ϵ RI-induced Degranulation and Cytokine Secretion in BMMC. Previous results have indicated that BMMC grown from Fyn or Gab2 deficient mice have defects in Fc ϵ RI-induced signaling resulting in impaired degranulation and cytokine release (27,28,30,33). Transfection of BMMC was therefore used to confirm the results observed with the two cell lines (Fig. 6). In BMMC within 24h of siRNA treatment the expression of Fyn decreased by 44% $\pm$ 8% while that of Gab2 decreased by 44% $\pm$ 6%; the

maximum reduction in expression of both proteins was at 48h post-transfection ($69\% \pm 13\%$ for Fyn and $72\% \pm 12\%$ for Gab2; Fig. 6, A and B). There was very similar reduction in the expression of Syk ($38\% \pm 13\%$ at 24h, $65\% \pm 15\%$ at 48h). The FcεRI-induced release of β-hexosaminidase was determined at both 24 and 48 hours post transfection. Syk knock down dramatically reduced β-hexosaminidase release ($52\% \pm 9\%$ at 24h, $77\% \pm 6\%$ at 48h, Fig. 6C). Degranulation did not change in BMMC treated with Fyn-specific siRNA regardless of Fyn knock down level (Fig. 6, A and B). However, the response was reduced in cells treated with Gab2-specific siRNA but this decrease did not fully correlate with knock down levels of Gab2. Thus, the β-hexosaminidase release decreased to a similar extent at 24h and 48h although Gab2 expression was lowest at 48h (Fig. 6).

BMMC were also used to determine the effect of reduced expression of Fyn and Gab2 on IgE-induced release of cytokines. For this, the release of TNF-α, IL-13 and CCL-4 (MIP-1β) was measured 48 hours after siRNA treatment. The dramatic decrease in Fyn expression did not affect the secretion of TNF-α or IL-13, but reduced the release of CCL-4 by $20\% \pm 3\%$ (Fig. 7). In contrast, the decrease in Gab2 resulted in reduced release of the three cytokines: TNF-α and IL-13 by $31\% \pm 13\%$, while $45\% \pm 18\%$ for CCL-4 (Fig. 7). These results with BMMC support the observations with the cell lines on the limited role of Fyn in degranulation but possible function of Gab2 in regulating cytokine release.

DISCUSSION

These experiments show that decrease in the expression of Syk, Fyn, and Gab2 have distinct consequences on FcεRI-mediated mast cell signaling. The results were consistent in BMMC and in two different cell lines, one of which is factor dependent. Decrease in Syk expression severely impaired immediate and late responses. In contrast, comparable decrease in Fyn expression had little or no effects on degranulation, cytokine release and signaling events such as phosphorylation of Akt and MAPKs. The decreased Gab2 expression had minimal effects on degranulation, but interfered

significantly with the late cellular responses. The results provide important insight on the relative contribution of these molecules to FcεRI signaling.

In mast cells, lack of Syk results in a complete loss of the FcεRI-induced signals that lead to degranulation and release of cytokines (8,9). The present results showed that the partial decrease in Syk expression still caused significant consequences in mast cells with a good correlation between the decrease in the level of this protein and the reduction in the cellular response. Syk knockdown decreased the activation of NFκB and NFAT and the PI3K pathway. These results demonstrate the critical role of Syk in mast cell responses.

Fyn contributes to signaling from receptors on T cells and plays a role in the activation, differentiation and survival of these cells (41-44). BMMC grown from Fyn^{-/-} mice proliferate normally but have defects in both c-kit and FcεRI-induced signaling. In these Fyn^{-/-} cells, c-kit stimulation results in normal PI3K and STAT3 activation, but defective p38 and JNK responses while FcεRI-activation results in impaired degranulation, and decreased cytokine release (27,28,45). In contrast, in the present experiments more than 80% reduction in Fyn expression had very minor if any effects on FcεRI-induced degranulation, cytokine release, MAPK phosphorylation or PI3K activation. These minor effects were statistically significant only at one time-point after stimulation and may not be biologically important because they were at time points that did not correlate with the maximum knockdown in Fyn expression. The difference between the present results and previous observations with Fyn^{-/-} BMMC could be because either a very small fraction of the Fyn in cells is required for signal transduction or cells from Fyn knockout mice have other defects due to adaptive changes during culture.

Gab2 has multiple binding sites for important signaling molecules including p85 PI3K, SHP2, Grb2, and Shc (27,29,30). In Gab2^{-/-} deficient mice there is decreased mast cell proliferation *in vitro* and lower number of mast cells in various tissues *in vivo* (31,32). BMMC from these mice have defective c-kit induced mast cell signaling and decreased FcεRI-induced degranulation that

were attributed to a reduction in PI3K activation with a parallel decrease in tyrosine phosphorylation of Akt (33). This Gab2 regulation of the PI3K pathway may play a role in controlling the development and proliferation of mast cells (31,33). Similar to these observations with BMMC, we found that decreased Gab2 expression reduced FcεRI-induced activation of the PI3K pathway as evident by decreased phospho-Akt levels, indicating that Gab2 was regulating the PI3K pathway in these cells. Reduced expression of this molecule in the present experiments caused a slight enhancement in degranulation in the MMC-1 cell line but a decrease in BMMC (Fig. 1C and Fig. 6). This difference could be due to variation in the activity of the PI3K pathway in the cell line compared to BMMC; experiments suggest that there is constitutive activation of this pathway in the MMC-1 cells (unpublished observations). Alternatively, the difference between the cells could be due to a change in the balance between positive and negative regulatory effects of Gab2. Many signaling molecules interact with Gab2; therefore the level of this protein in cells could have both positive and negative effects. The enhancement or inhibition of degranulation could depend on the balance of these interactions of Gab2 with these other proteins.

Still, our results indicate that FcεRI-induced late responses are regulated by Gab2; the reduced expression of this protein inhibited late responses such as MAPK phosphorylation and cytokine release. Gab2 knockdown had no effect on receptor-induced NFAT activation but decreased the NFκB activity. This was paralleled with decreased release of cytokines in BMMC and the cell lines; confirming the role of this adaptor in cytokine-related signaling (31,33).

Studies with Fyn deficient BMMC suggest an interaction between Fyn and Gab2 (27). In Fyn deficient cells there is decreased tyrosine phosphorylation of Gab2 although this phosphorylation is downstream of Syk (27,32). Gab2 may play a role in granule translocation to the plasma membrane, through a calcium-independent mechanism that also involves Fyn and small GTPase RhoA (34). However, we did not observe any changes in the phosphorylation of Gab2 at two sites after decreased Fyn expression (Fig. 3C; Supplemental Fig. 3C).

The present results have important differences

from observations with BMMC derived from mice with knockout of the same proteins. There can be several possible reasons for these differences. The amount of a protein required for signaling in these pathways may be in such excess that even >80% decrease in expression as was achieved in the present experiments will not be a limiting factor in signal transduction. This clearly was not the case for both Syk and Gab2 where the decreased protein levels had effects on downstream events. Another possibility is that there could be changes in molecules other than the ones under study that could be modifying signal transduction. For example, the knockout of a gene could result in compensatory changes in expression or activity of other proteins. This would be more of a problem in long-term experiments such as in BMMC, compared to the short-term knockdown with siRNA. In Lyn negative BMMC there is decreased expression of TRPC4 protein that may explain the delayed calcium response of these cells (22,46). Another factor could be the genetic background of the cells; it has been observed that knockdown of Lyn has different effects on mast cell signaling depending on the genetic background of the mice, so that the observed changes may be due to molecules other than Lyn (47). On the other hand, established cell lines could have mutations that influence signal transduction; this could especially be a factor in tumor derived cell lines. To obviate this problem, we used BMMC besides the two different cell lines; MMC-1 which is tumor derived whereas the MC9 are factor dependent. The results with all three cell types were very similar strongly suggesting that the observed changes were due exclusively to the applied siRNA treatment.

In summary, our findings indicate that Syk regulates immediate and late cellular responses after FcεRI activation in mast cells while Gab2 controls late responses. There were only minor effects in early or late responses by up to 80% decreased expression of Fyn. Therefore, pharmacological inhibitors of Syk would be effective in reducing both early and late responses, while those that act on Gab2 would be effective on late responses. In contrast, inhibition of Fyn would not be effective in regulating the mast cell response.

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FIGURES LEGEND

FIGURE 1. Decreased expression of Syk but not Fyn or Gab2 reduces FcεRI-induced degranulation. *A*, MMC-1 cells were treated with siRNA specific for Syk, Fyn or Gab2 (indicated by “T”) or with siCONTROL (indicated as “C”) and expression of the target proteins was determined by immunoblotting 24h, 48h and 72h after transfection. The fraction of the protein left in the transfected cells compared to the controls was determined by densitometry after normalization for gel loading from blots probed with anti-FcεRIβ. One representative experiment is shown. *B*, Summary of the change in the expression level of these proteins; results are mean ±SD, n=6. *C*, Antigen-induced β-hexosaminidase release in transfected MMC-1 cells. Degranulation was determined at the indicated times in IgE-sensitized cells. Percent of total β-hexosaminidase release in control transfected cells was 39%±3% at 24h; 33%±6% at 48h; 28%±5% at 72h. Results are mean±SD (n=6). *D*, Antigen dose response of β-hexosaminidase release at 48h after transfection. IgE-primed MMC-1 cells were stimulated with the indicated concentrations of antigen. β-hexosaminidase release is presented as percent of total cellular enzyme content (mean±SD; n=6). The release curves with Syk and Gab2 siRNA were significantly different than the control ($p \leq 0.001$ paired two-sided Student t-test).

FIGURE 2. Decreased expression of Fyn or Gab2 has minor effects on FcεRI-induced activation of NFκB and NFAT. *A*, MC9 NFκB-GFP cells or *C*, MC9 NFAT-GFP cells were treated with siRNA specific for Syk, Fyn or Gab2 (indicated by “T”) or with siCONTROL (indicated by “C”) and expression of the target proteins was determined by western blotting at the indicated times. The fraction of the protein left in the transfected cells compared to the controls as determined by densitometry is indicated. One representative experiment is shown. *B*, The activity of NFκB (n=9) and *D*, the activity of NFAT (n=10) was determined by FACS analysis at the indicated times after transfection and are expressed as percentage of the control (dotted line). Bars indicate SD.

FIGURE 3. Gab2 but not Fyn regulated IgE-dependent Akt phosphorylation. *A*, **Gab2 regulates Akt phosphorylation.** MC9 cells were transfected, starved of SCF for 24h and then stimulated with antigen for the indicated times. Lysates were analyzed by immunoblotting sequentially with antibodies for phospho-Akt Ser473 (anti-pAkt) and Akt. *B*, Changes in phospho-Akt are expressed as percent of the control (dotted line) in cells transfected with the indicated siRNA. Results are mean±SD (n=4). *C*, Fyn-independent phosphorylation of Gab2. MC9 cells were treated with Fyn-specific siRNA or siCONTROL then stimulated with antigen at 48h after transfection. The resolved proteins were immunoblotted with antibodies for Gab2 phospho-Ser159, Gab2 phospho-Tyr452 and Gab2. One representative experiment out of three is shown.

FIGURE 4. Gab2 but not Fyn regulated IgE-induced MAPK phosphorylation. IgE-primed MC9 cells were stimulated with antigen for the indicated times 48h after the siRNA treatment. Resolved proteins were blotted as follows: *A*, with antibodies for phospho-Erk (Thr202/Tyr204) and Erk; *C*, with antibodies for phospho-p38 (Thr180/Tyr182) and p-38; *E*, with antibodies for phospho-JNK (Thr183/Tyr185) and JNK. Percent decrease in the phosphorylation levels of Erk, n=4 (*B*), or p38, n=4 (*D*) and JNK, n=2 (*F*) is expressed as percentage of the control (dotted line). Bars indicate SD.

FIGURE 5. TNF-α secretion was inhibited by reduced expression of Gab2 but not Fyn. MMC-1 (*A*) or MC9 cells (*C*) were transfected with siRNA specific for Fyn or Gab2 or with siCONTROL. After 48h cells were stimulated with antigen for 3h. Amount of released TNF-α is expressed as percent of the release in the control cells (TNF-α release in control treated cells was 1394±182 pg/ml in MMC-1 and 170±76 pg/ml in MC9). Each cell line was tested twice. Representative immunoblots for MMC-1 (*B*) and MC9 (*D*) are shown.

FIGURE 6. IgE-mediated degranulation in BMMC with reduced expression of Syk, Fyn or Gab2. *A*, Efficient siRNA-induced decrease in expression of Syk, Fyn and Gab2 in BMMC. Knock down levels were determined at the indicated times by Western blotting. The protein left in the transfected cells compared to the controls was calculated by densitometry after normalization for gel loading and expressed as percent of controls. Results are mean $\pm$ SEM; for Syk n=4 (24h and 48h); for Fyn n=12 (24h), n=18 (48h); for Gab2 n=10 (24h), n=18 (48h). *B*, A representative immunoblot showing decreased expression of target proteins 48h after transfection. *C*, Ag-induced β -hexosaminidase release in transfected BMMC. IgE-primed BMMC were stimulated for 45 min with antigen. Percent of total β -hexosaminidase release in control transfected cells was 18% $\pm$ 6% at 24h and 19% $\pm$ 7% at 48h. Results are mean $\pm$ SEM.

FIGURE 7. IgE-mediated release of cytokines in BMMC with reduced expression of Fyn or Gab2. BMMCs were transfected with siRNA specific for Fyn, Gab2 or with siCONTROL, then 48h later IgE-primed cells were stimulated with antigen for 3h. Amounts of released TNF- α (*A*, n=8), IL-13 (*B*,; n=8) and CCL-4 (*D*; n=7) was determined by ELISA.

Figure 1

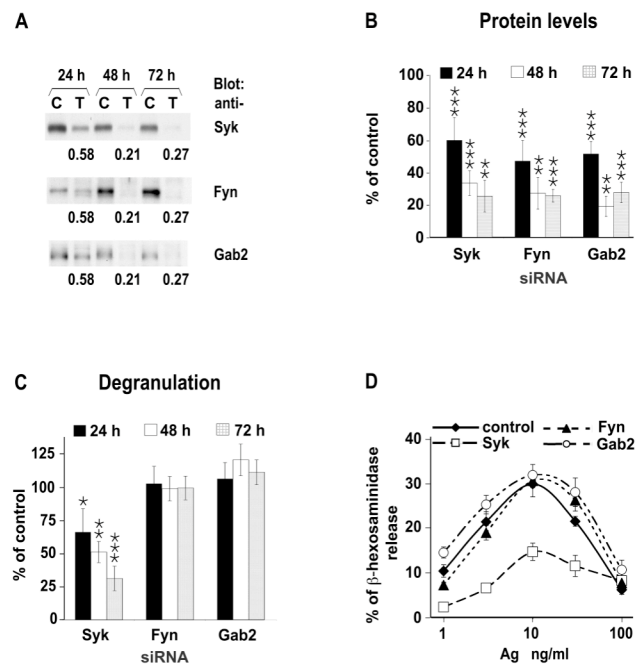


Figure 2

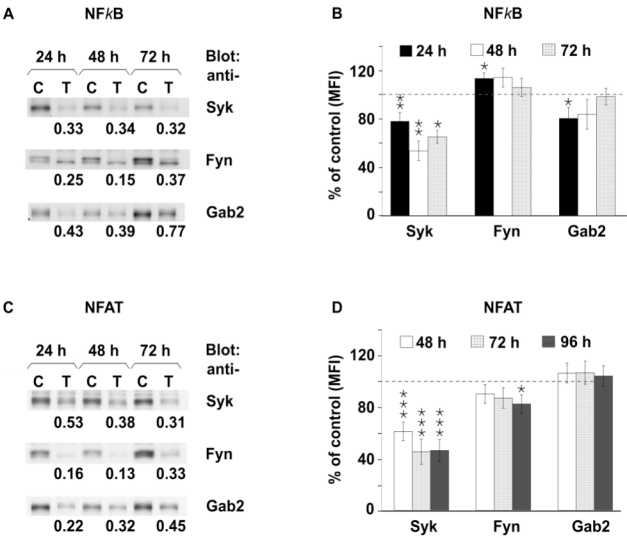


Figure 3

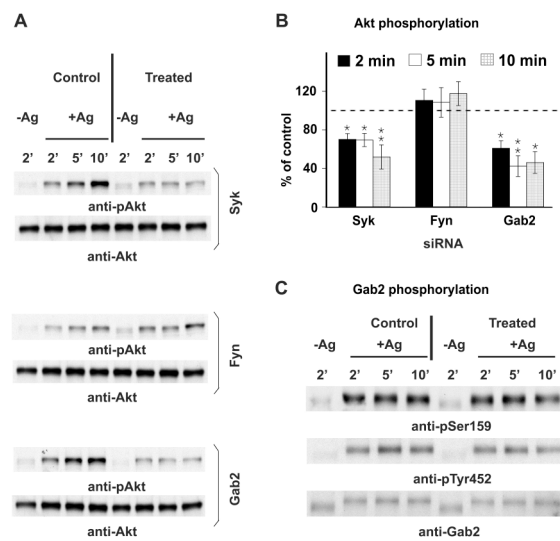


Figure 4

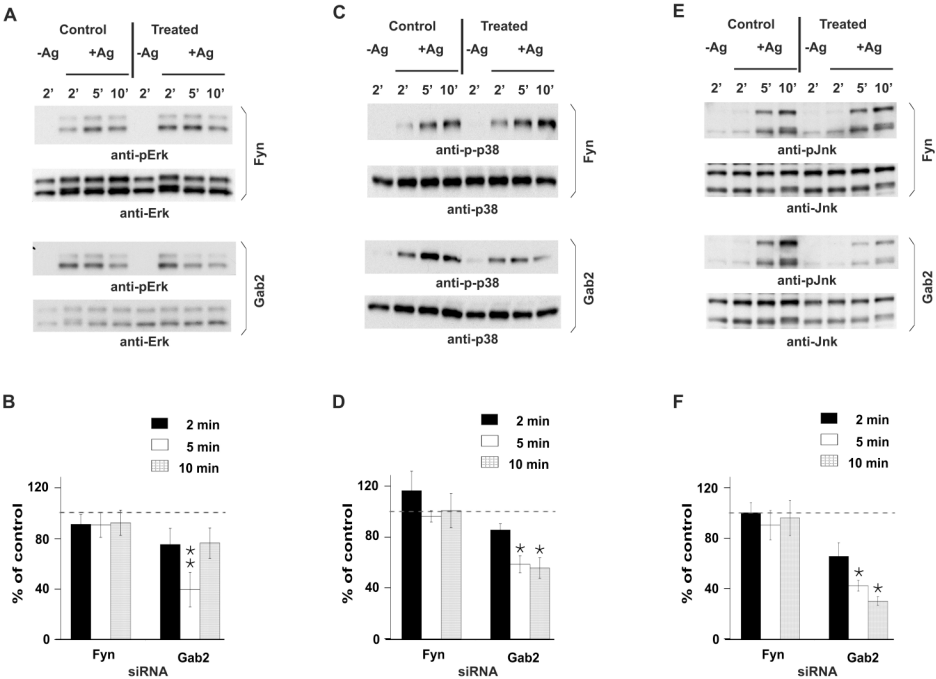


Figure 5

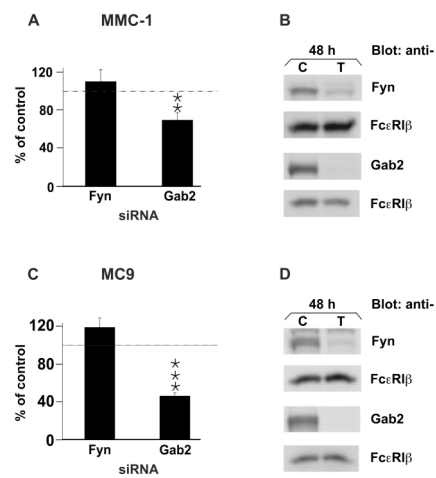


Figure 6

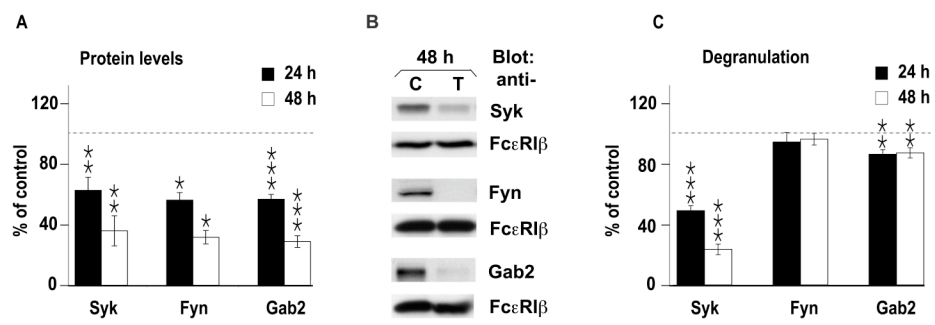
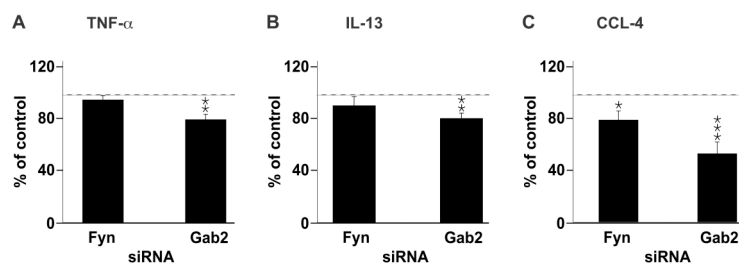


Figure 7



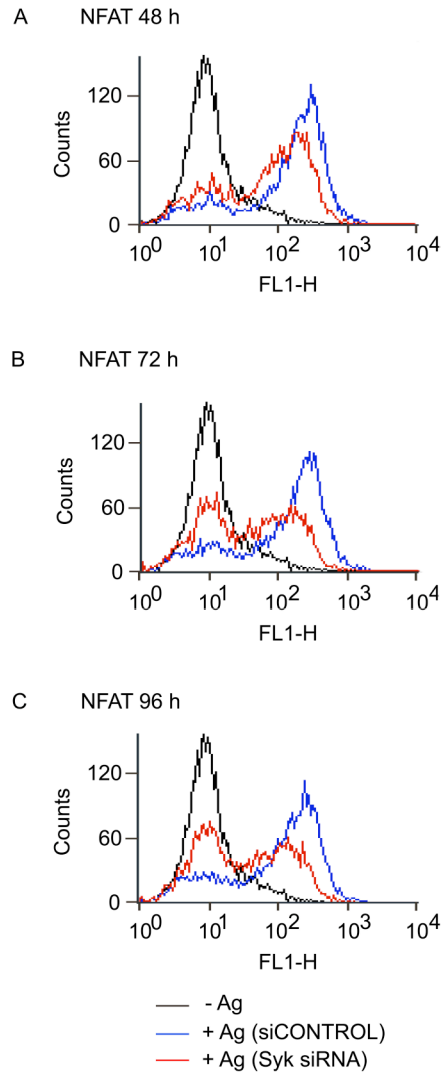
Supplemental Materials and Methods

Supplemental Table 1. siRNA pools used in these experiments

duplex (5)	J-041084-05 SYK	sense	GAACUGAGGCGUUCGCAAUUUU
		antisense	5'-PAAUUGCGAAGCCUCAGUUCUU
duplex (6)	J-041084-06 SYK	sense	GGAAUAAUCUCAAGGAUCAUU
		antisense	5'-PUGAUCCUUGAGAUUAUUCCUU
duplex (7)	J-041084-07 SYK	sense	CAGCAGAACAGGCACAUUAUU
		antisense	5'-PUAAUGUGCCUGUUCUGCUGUU
duplex (8)	J-041084-08 SYK	sense	CCGAAUGCAUCAACUACUAUU
		antisense	5'-PUAGUAGUUGAUGCAUUCGGUU
duplex (5)	J-040112-05 FYN	sense	GGUGCGAAGUUUCCCAUUAUU
		antisense	5'-PUAAUGGGAAACUUCGCACCUU
duplex (6)	J-040112-06 FYN	sense	GCACGUGACUCGUUGUUUCUU
		antisense	5'-PGAAACAACGAGUCACGUGCUU
duplex (7)	J-040112-07 FYN	sense	UAACUGUGGUUUCAUCAAGUU
		antisense	5'-PCUUGAUGAAACCACAGUUAUU
duplex (8)	J-040112-08 FYN	sense	CCACGUCAAACAUAUAAAUU
		antisense	5'PUUUAUAAUGUUUGACGUGGUU
duplex (5)	J-041087-06 GAB2	sense	GGAAAUCGCCUCCCGAGAAUU
		antisense	5'-PUUCUCGGGAGGCGAUUCCUU
duplex (6)	J-041087-07 GAB2	sense	CCUGUGAGACCUACGAGUAUU
		antisense	5'-PUACUCGUAGGUCUCACAGGUU
duplex (7)	J-041087-07 GAB2	sense	CAGCAAAGACCUCCAAUCAUU
		antisense	5'-PUGAUUGGAGGUCUUUGCUGUU
duplex (8)	J-041087-08 GAB2	sense	GAAACAACACUGUCAUCGAUU
		antisense	5'-PUCGAUGACAGUGUUGUUUCUU

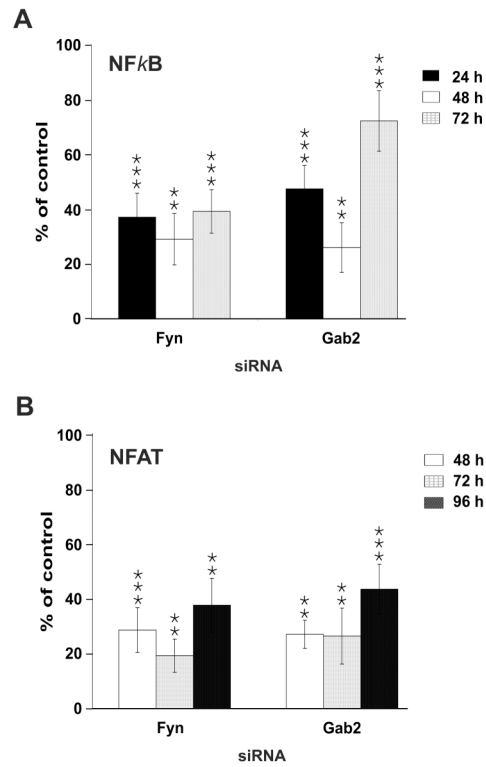
Supplemental Results

Supplemental Figure 1



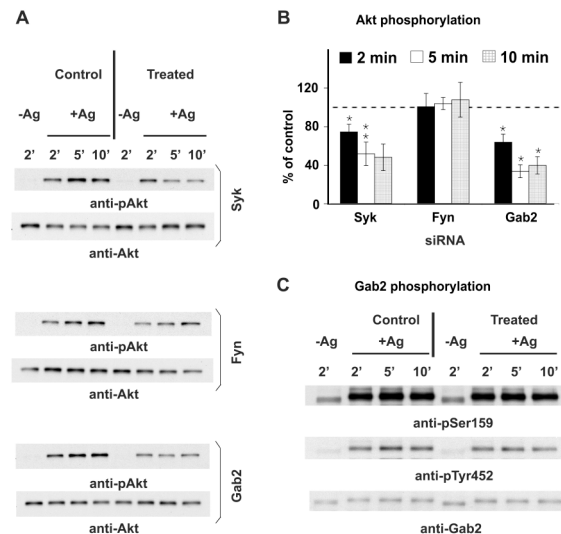
FACS analysis of NFAT activity in mast cells treated with Syk-specific siRNA. MC9 NFAT-GFP cells were treated with siRNA for Syk (red line) or siCONTROL (blue line). The cells were primed with IgE for 24h, then cultured for another 24h with or without antigen and the fluorescence of the GFP reporter was used to determine NFAT activity at 48h (A), 72h (B) and 96h (C) post-transfection. Basal GFP fluorescence of non-stimulated cells is also indicated (black line). One representative experiment out of ten is shown.

Supplemental Figure 2



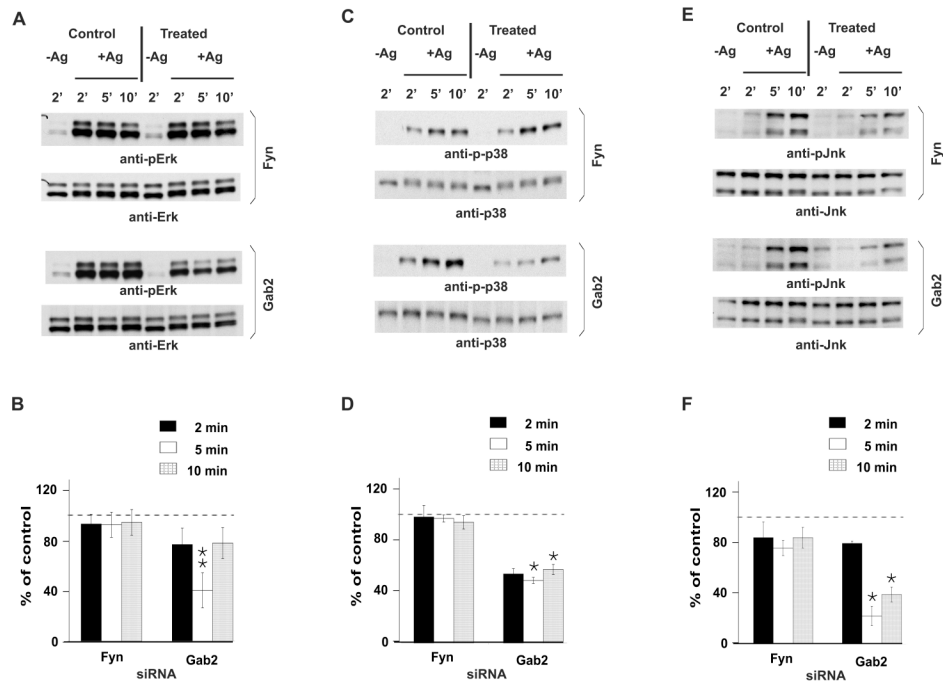
Efficient decrease in Fyn and Gab2 protein levels by siRNA treatment. The decrease in the expression of the target proteins was determined at the indicated times by Western blotting. The protein left in the transfected cells compared to the controls was calculated by densitometry after normalization for gel loading and expressed as percent of controls. Results are mean $\pm$ SD; (A) MC9 NFκB, n=9; (B) MC9 NFAT, n=10.

Supplemental Figure 3



Gab2 regulated IgE-dependent Akt phosphorylation and was phosphorylated at sites independently of Fyn (MMC-1 cells). *A*, Gab2 regulates Akt phosphorylation. MMC-1 cells were transfected with siRNA, starved of SCF for 24 h and then stimulated with antigen for the indicated times. Lysates were analyzed by immunoblotting sequentially with antibodies for phospho-Akt Ser473 (anti-pAkt) and Akt. Similar results were observed in 4 independent experiments. *B*, Changes in phospho-Akt is expressed as percent of the control (dotted line; mean $\pm$ SD, $n=4$) in cells transfected with the indicated siRNA. *C*, Fyn-independent phosphorylation of Gab2. MMC-1 cells treated with Fyn-specific siRNA or siCONTROL were then stimulated with antigen at 48h after transfection. The resolved proteins were immunoblotted with antibodies specific for Gab2 phospho-Ser159, Gab2 phospho-Tyr452 and Gab2. One representative experiment out of four is shown.

Supplemental Figure 4



Gab2 but not Fyn regulated IgE-induced MAP kinase phosphorylation (MMC-1 cells). IgE-primed MMC-1 cells were stimulated with antigen for the indicated times 48h after the siRNA treatment. Resolved proteins were sequentially blotted as follows: with antibodies for phospho-Erk (Thr202/Tyr204) and Erk in *A*; with antibodies for phospho-p38 (Thr180/Tyr182) and p-38 in *C*; with antibodies for phospho-Jnk (Thr183/Tyr185) and Jnk in *E*. Time-dependent pattern and level of phosphorylation of these MAP kinases were similar in NF κ B-GFP and NFAT-GFP cells. Percent decrease in the phosphorylation levels of MAP kinases is expressed as percentage of the control (dotted line) for in (*B*) Erk, *n*=5; (*D*) p38, *n*=4 and (*F*) Jnk, *n*=2. Bars indicate SD.