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Src-like adaptor protein (SLAP) is upregulated in antigen-stimulated mast cells and acts as a negative regulator

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Abstract

Our studies in the RBL-2H3 mast cell line suggest that responses to antigen (Ag) are negatively modulated through upregulation of Src-like adaptor protein (SLAP). Ag stimulation of RBL-2H3 cells leads to increased levels of SLAP (but not SLAP2) transcripts and protein over a period of several hours. The effects of pharmacologic inhibitors indicate that the upregulation of SLAP is dependent on multiple signaling pathways. Knockdown of SLAP with anti-SLAP siRNA is associated with enhanced phosphorylation of Syk, the linker for activation of T cells (LAT), phospholipase Cγ, MAP kinases, and various transcription factors. Production of IL-3 and MCP-1, but not degranulation, is also enhanced. The upregulation of SLAP may thus serve to limit the duration of cytokine production in Ag-stimulated cells.

Keywords

Mast cells; Antigen; Src-like Adaptor Protein; Inhibitory regulator; Signaling; Cytokines

1. Introduction

Stimulation of mast cells via the IgE receptor (FcεRI) results in the activation of phospholipase (PL) C and D as well as the MAP kinases, Erk, JNK, and p38 MAP kinase (Rivera and Gilfillan, 2006; Gilfillan and Tkaczyk, 2006). Activated PLC and PLD, in turn, produce messenger molecules that activate protein kinase (PK) C and mobilize Ca^{2+} from intracellular and extracellular sources to generate necessary signals for degranulation with the release of preformed inflammatory mediators (Ozawa *et al.*, 1993; Peng and Beaven, 2005). These signals along with the activation of the MAP kinases lead to rapid activation of PLA_2, which is essential for production of inflammatory eicosanoids (Hirasawa *et al.*, 1995a; Hirasawa *et al.*, 1995b), and the activation of downstream transcription factors that initiate cytokine gene transcription (Hundley *et al.*, 2004; Qiao *et al.*, 2006). Collectively these events promote the typical hypersensitivity reactions to antigen (Ag).

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The proximal components of FcεRI-mediated signaling pathways that lead to the responses noted above include the cytosolic Src kinases Lyn and Fyn, Syk tyrosine kinase, and the transmembrane adaptor proteins linker for activation of T cells (LAT) and non-T-cell activation linker (NTAL) which, when phosphorylated by these kinases, serve as docking sites for the assembly of other adaptor and signaling molecules (Rivera and Gilfillan, 2006; Gilfillan and Tkaczyk, 2006). In addition, the production of phosphatidylinositol 3,4,5-*tris*phosphate by phosphatidylinositol 3-kinase provides additional nucleation sites for recruitment and activation of other signaling molecules at the plasma membrane. An early signaling event is the phosphorylation of FcεRI by Lyn and recruitment of Syk by phosphorylated FcεRI. Syk, in turn, is essential for downstream phosphorylation of LAT and activation of PLCγ.

Mast cells also possess mechanisms for negatively regulating cell activation (Siraganian, 2003; Molfetta *et al.*, 2007). These mechanisms are most pronounced at supraoptimal concentrations of Ag (Xiao *et al.*, 2005) or on co-ligation of FcεRI with inhibitory receptors that contain the immunoreceptor tyrosine-based inhibitory motif (ITIM) (Ott and Cambier, 2000; Li and Yao, 2004). Inhibitory signals in Ag stimulated cells may be orchestrated through Lyn (Xiao *et al.*, 2005) or the ubiquitin ligases c-Cbl and Cbl-b to induce ubiquitination and degradation of FcεRI subunits (Paolini *et al.*, 2002), Lyn (Kyo *et al.*, 2003; Qu *et al.*, 2004), and Syk (Ota and Samelson, 1997; Paolini *et al.*, 2002). Other mechanisms include the engagement of the inositol phosphatases, phosphatase and tensin homologue deleted on chromosome ten (PTEN) and SH2 domain-containing inositol polyphosphate 5-phosphatase (SHIP) both of which reduce levels of phosphatidylinositol 3,4,5-trisphosphate (Kimura *et al.*, 1997a; Furumoto *et al.*, 2006). The SH2 domain-containing protein tyrosine phosphatases, SHP-1 and SHP-2, are recruited by phosphorylated FcεRI and attenuate mast cell activation by dephosphorylating various components of the signaling pathways (Kimura *et al.*, 1997b). A mechanism that warrants further investigation is the induction of inhibitory regulators such as Dok1 (downstream of tyrosine kinase-1), MAP kinase phosphatase-1 also known as dual specificity protein phosphatase-1 (DUSP1) and Src-like adaptor protein (SLAP). Dok1 has been implicated in the repressive actions of the ITIM-bearing inhibitory receptors, FcγRIIB (Ott *et al.*, 2002; Kepley *et al.*, 2004) and the mast function-associated antigen (MAFA) (Abramson and Pecht, 2002). Dok1 (Hiragun *et al.*, 2005), DUSP1 (Kassel *et al.*, 2001; Jeong *et al.*, 2003), and SLAP (Hiragun *et al.*, 2006; Park and Beaven, 2009) are also upregulated by low concentrations of dexamethasone and, in this manner, contribute to the inhibitory actions of this and other glucocorticoids in mast cells.

The inhibitory regulators target specific components of the signaling pathways. Dok1, for example, negatively regulates Ras by activating the Ras GTPase activating protein and thus inhibits the entire Ras/Raf1/Erk/PLA_2 pathway in Ag-stimulated cells (Hiragun *et al.*, 2005). DUSP1 is one member of a family of DUSPs that dephosphorylate and inactivate MAP kinases (Lang *et al.*, 2006; Owens and Keyse, 2007). SLAP family members, SLAP (Sosinowski *et al.*, 2000; Tang *et al.*, 1999; Dragone *et al.*, 2006) and SLAP2 (Holland *et al.*, 2001; Pandey *et al.*, 2002; Loreto *et al.*, 2002), interact with components of the T and B cell receptor signaling complexes and thus negatively regulate cellular signaling. The SLAPs possess SH2 and SH3 domains that are homologous to those in Src kinases and binding partners include Src kinase, ZAP70, CD3ζ, and Cbl (Pandey *et al.*, 1995; Sosinowski *et al.*, 2000; Tang *et al.*, 1999; Holland *et al.*, 2001; Pandey *et al.*, 2002; Loreto *et al.*, 2002). SLAP co-opts c-Cbl to down-regulate expression of T and B cell receptors (Loreto *et al.*, 2002; Dragone *et al.*, 2006; Myers *et al.*, 2006). SLAP and SLAP2 also down-regulate growth factor receptor mediated signaling in fibroblasts in a Src kinase and c-Cbl dependent manner (Pakuts *et al.*, 2007; Sirvent *et al.*, 2008). In dexamethasone-treated mast cells, SLAP negatively regulates Syk (a cognate of ZAP70) and Syk-dependent signaling events such as the phosphorylation of LAT and PLCγ2 as well as the mobilization of Ca^{2+} ions (Hiragun *et al.*, 2006). As a consequence, increased

expression of SLAP, whether induced by dexamethasone or by gene transfection, results in diminished Ag-induced degranulation and release of arachidonic acid (Hiragun *et al.*, 2006).

We have investigated whether or not the inducible inhibitory regulators play a role in regulating responses to Ag itself. Preliminary investigations indicated that Ag stimulation of the RBL-2H3 mast cell line resulted in substantial increases in levels of SLAP mRNA, a small transient increase in DUSP1 mRNA, but no increase in levels of DOK1. Here we have focused on the consequences of the induction of SLAP because it suppresses a primary signaling event in mast cells, namely the activation of Syk (Hiragun *et al.*, 2006). We find that the upregulation of SLAP by Ag not only suppresses the phosphorylation of Syk but also phosphorylation of MAP kinases and downstream transcription factors in addition to cytokine production.

2. Materials and methods

2.1. Reagents and antibodies

Reagents were obtained from the following sources: Media and culture reagents and Trizol from Invitrogen/GIBCO (Carlsbad, CA); dinitrophenyl-human serum albumin (DNP-HSA), Cyclosporin A and mouse anti-DNP IgE, Sigma (St. Louis, MO); polyclonal antibodies against SLAP (C-19), Syk and PLCγ2 from Santa Cruz (Santa Cruz, CA); antibody against Dok1 from Abcam (Cambridge, MA); all other antibodies from Cell Signaling (Danvers, MA); siRNA against SLAP from Dharmacon (Chicago, IL); Bay11-7082, PD98059, SB203580, SP600125, LY294002, Ro318220 from Calbiochem (San Diego, CA); ELISA kits for TNFα, IL-3, and MCP1 from Biosource (Camarillo, CA); and FITC-conjugated mouse IgE monoclonal isotype control from BD Pharmingen (San Jose, CA).

2.2. Cell culture and transient transfection of siRNAs

RBL-2H3 cells were maintained in minimal essential medium (MEM) supplemented with 15% fetal calf serum, 2 mM $_{L}$-glutamine, and an antibiotic-antimycotic solution. For transfection with siRNAs, cells were detached with trypsin-EDTA solution and 2×10^6 cells were pelletted and suspended in 100 μl of Nucleofector Solution R (Amaxa, Walkersville, MD). The suspension was mixed with 200 pmoles of ON-Targetplus SMARTpool siRNA against SLAP (Dharmacon, Lafayette, CO) and transfected by electroporation program T-20 (Amaxa, Walkersville, MD). The transfected cells were used after 48 h for each experiment.

2.3. Measurement of degranulation, release of arachidonic acid, and production of cytokines

Transfected cells were plated in 24-well (0.1×10^6 cells/1 ml/well) or 6-well (0.25×10^6 cells/2.5 ml/well) plates for measurement of β-hexosaminidase and cytokines respectively. Cells were incubated for 24 h at 37°C. The culture medium was then replaced with fresh medium containing 50 ng/ml anti-DNP-IgE and incubated for a further 24 h. The cultures were washed twice with glucose saline/PIPES buffer (Choi *et al.*, 2002) and stimulated with 50 ng/ml DNP-HSA in saline/PIPES buffer for 20 min for assay of the granule marker β-hexosaminidase in medium and cells (Ozawa *et al.*, 1993) or for the indicated times in complete growth medium for measurement of cytokines by use of ELISA kits (Qiao *et al.*, 2006). Data were expressed as a percent of cellular β-hexosaminidase that was released into the medium or total cytokine released into medium.

2.4. Western blot analysis

Cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed in lysis buffer (25 mM Tris-HCl pH7.5, 150 mM NaCl, 1% nonidet P-40, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml aprotinin, and 2 μg/ml pepstatin)

and left for 30 min on ice. Proteins were separated in 4–12% gradient SDS-PAGE gels and transferred to PVDF membrane. Immunoreactive proteins were visualized by use of horseradish peroxidase-conjugated secondary antibodies and chemiluminescence (Millipore, Billerica, MA).

2.5. Measurement of mRNAs

Cell cultures were stimulated with Ag as described above for the assay of cytokines. They were washed twice with PBS and then lysed with 1 ml Trizol. Total RNA was purified from cells of which 1 μg was used for synthesis of cDNA according to the manufacturers' protocols (Invitrogen, Carlsbad, CA). Transcript levels of Dok1 and SLAP, and 18S rRNA (an internal control to calculate fold induction) were assayed by real-time PCR with the following primers and probes: SLAP, ATCCAGTTTGCAGGAAGGTCCAGA, TAGGATGCGATGCTTTCCCGAAGA, TaqMan® probe FAM-AGAGAACCCACTCAGAGTGGACGAAT –TAMRA; Dok1, TCTTTCAGGCAGTTGAGGCTGCTA, GCACGTGCCCATACAAATCCCAAT; IL-3 TCCTGATGCTCTTCCACC, TCGCAGCTGCAGGAATACAACACT; MCP1, TGGCAAGATGATCCCAAT, AGGTGGTTGTGGAAAAG; TNFα, TTGCCACTTCATACCAGGAGAA, TCACAGAGCAATGACTCCAA.

2.6. Measurement of FcεRIα on cell surface with FITC-labeled IgE

Transfected cells were detached from culture plates 48 h after transfection by repeated pipetting and then washed with Dulbecco's PBS containing 0.1% sodium azide (FACS buffer). For flow cytometric analysis, 1 μg of FITC-conjugated mouse IgE was incubated with 1×10^6 cells in 100 μl FACS buffer for 1 h at 4°C. Cells were washed with FACS buffer and cell-bound IgE was measured by flow cytometry (Becton Dickinson, FACSCalibur). Preliminary experiments had indicated maximal and saturable binding of the labeled IgE to cells under the conditions described here.

3. Results

3.1. Ag upregulates SLAP through multiple signaling pathways

Examination of expression levels of known inhibitory regulators in Ag-stimulated mast cells revealed a more than ten-fold increase in expression of SLAP mRNA (Fig. 1A) whereas the expression of Dok1 was unaffected (Fig. 1B). SLAP mRNA levels reached a maximum within 2 h to 3 h of addition of Ag and then gradually declined towards basal levels but were still elevated at 6 h. The increase in mRNA was accompanied by a substantial increase in SLAP protein reaching a maximum at 3 h (inset, Fig. 1A). The increase in SLAP mRNA was evident with concentrations of Ag that were capable of stimulating degranulation and the concentration-response curves were similar for both responses (Fig. 1C). As in a previous study (Park and Beaven, 2009), SLAP2 mRNA was undetectable in either resting or stimulated cells RBL-2H3 cells when examined with a variety of primers (data not shown).

Studies with various inhibitors indicated that production of SLAP mRNA was dependent on p38 MAP kinase, JNK, phosphatidylinositol 3-kinase, calcineurin, and PKC, all of which are activated by Ag and inhibited by respectively, SB202190, SP600125, LY204002, cyclosporin A, and Ro-31 8220 in RBL-2H3 cells (Andrade *et al.*, 2004; Hundley *et al.*, 2004; Qiao *et al.*, 2006). All of these inhibitors suppressed the Ag-induced increase in SLAP mRNA (Fig. 2). The MEK and NFκB inhibitors, PD 98059 and BAY 11-7082, in contrast, had minimal effects on production of SLAP mRNA (Fig. 2) to suggest that the ERK1/2 and NFκB pathways were not essential for transcriptional activation of the SLAP gene. Several transcription factors are activated through JNK, p38 MAP kinase, protein kinase C, and calcineurin by Ag (Razin *et al.*, 1994; Qiao *et al.*, 2006; Tkaczyk *et al.*, 2006). These include components of activator

protein-1 (AP-1) complex, ATF2, and NFAT. Therefore, the enhanced production of SLAP transcripts is probably regulated through multiple signaling pathways and transcription factors although ERK and NFκB do not appear to be involved.

3.2. Knock down of SLAP with siRNA enhances expression of FcεRI

Cells transfected with anti-SLAP siRNA contained ~ 40% less SLAP than control RNA-transfected cells. Following Ag stimulation the difference between the two sets of cells ranged from 40% to 70% for all experiments (see data in Fig. 3A for a typical experiment). As determined by immunoblotting, Ag stimulation resulted in a modest decline in expression of FcεRIγ subunits over the course of six hours (~25% for three experiments) but no consistent difference in rates of decline could be detected between control and siRNA-treated cells (data not shown). However, the capacity of cells to bind FITC-labeled IgE, as determined by flow cytometry, was increased slightly by knock down of SLAP. The average additional binding on anti-SLAP siRNA-treated cells was 14% with values ranging from 9% to 17% in five experiments (data not shown).

3.3. Effects of SLAP deficiency on signaling events

As noted above, knockdown of SLAP by siRNA diminished SLAP levels in both basal and Ag-stimulated cells (Fig. 3A). The reduction in SLAP was associated with enhanced tyrosine phosphorylation of Syk and its downstream targets LAT and PLCγ2 following Ag stimulation (Fig. 3A). This was also true for the phosphorylation of ERK, JNK, and p38 MAP kinase (Fig. 3B). The enhancement of these early signaling events was reflected in enhanced activation of downstream transcription factors as indicated by increased phosphorylation of c-Jun, ATF2, and NFκB and possibly increased expression of NFB (Fig. 3C). The most notable enhancement of phosphorylation was that of c-Jun and NFκB one to two hours after the addition of Ag.

3.4. Effects of knock-down of SLAP on degranulation and cytokine production

The reduction in SLAP mRNA in anti- SLAP siRNA-treated cells (Fig. 4A) had no discernable affect on the release of the granule marker, β-hexosaminidase (Fig. 4B). In contrast, production of IL-3 and monocyte chemoattractant protein-1 (MCP-1) transcripts (Fig. 4C and D) and protein (Fig. 4E and F) were enhanced by knockdown of SLAP. The attenuated increase in SLAP transcripts and enhanced increase in IL-3 and MCP-1 transcripts exhibited an almost reciprocal relationship with the differences being most apparent 2 and 3 h after addition of Ag (Fig. 4A, C, and D). However, Ag stimulated production of TNFα was not significantly enhanced (data not shown) to indicate that the negative effects of SLAP did not extend to all cytokines.

4. Discussion

We find that stimulation of RBL-2H3 cells with Ag leads to increased transcription of the SLAP gene (*Sla-1*) and a substantial increase in SLAP protein (Fig. 1A). The extent of increase in SLAP mRNA is dependent on concentration of Ag (Fig. 1C). At optimal concentrations of Ag the increase (15 to 30 fold) is equal to or greater than that observed in dexamethasone-treated RBL-2H3 cells which typically exhibit about a tenfold increase in SLAP mRNA 12 h after addition of dexamethasone (Hiragun *et al.*, 2006). This upregulation is dependent on activation of several signaling pathways that appear to include PKC, JNK, p38 MAP kinase, and calcineurin (Fig. 2) and presumably on the activation of transcription factors that lie downstream of these enzymes. The increase in SLAP is apparent within 60 min of stimulation and reaches a maximum by two to three hours (Fig. 1A), a time-course that might have little impact on degranulation (Fig. 4B) but sufficiently rapid to reduce production of some cytokines and chemokines at later stages of Ag stimulation (Fig. 4E and F).

An ambiguity is that the reduction of basal levels of SLAP by siRNA in resting RBL-2H3 cells is obviously sufficient to enhance early Syk-dependent signaling events (Fig. 3A) but not degranulation (Fig. 4B). Previous studies have shown that RBL-2H3 cells generate stimulatory signals in excess of that required for degranulation (Maeyama *et al.*, 1986) and it is possible that signals of additional strength would have little impact on degranulation. Also, maximal rates of degranulation occur at concentrations of free Ca^{2+} (~300 nM) well below those normally observed in optimally stimulated cells (Beaven and Ozawa, 1996). However, the relatively robust phosphorylation of c-Jun and NFκB at one and two hours (Fig. 3C) does apparently result in augmented production of IL-3 and MCP-1 (Fig. 4C to F) in SLAP-deficient cells.

Previous studies with dexamethasone and overexpressed SLAP in RBL-2H3 cells demonstrated that SLAP suppressed activation of Syk and Syk-dependent signaling events such as the phosphorylation of LAT and PLCγ2 as well as mobilization of Ca^{2+} (Hiragun *et al.*, 2006). The present results with siRNA against SLAP imply that SLAP represses not only activation of these signaling events but also the activation of MAP kinases and transcription factors and, as a consequence cytokine production, thus limiting the duration of their production in Ag-stimulated mast cells. For example, repression of induction of SLAP by siRNA enhances phosphorylation of the AP-1 component c-Jun, ATF-2, and NFκB (Fig. 3C) as well as the production of IL-3 and MCP-1 (Fig. 4C to D). These and our previous data (Hiragun *et al.*, 2006) suggest that SLAP can negatively regulate Syk and signaling pathways down to the nuclear and transcriptional levels. A relevant situation is that Syk −/− BMMC exhibit similar defective signaling and physiologic responses to Ag which include impaired activation of Erk2, JNK, Ca^{2+}/calcineurin-dependent dephosphorylation of NFAT, and production of various cytokines (Costello *et al.*, 1996).

The upregulation of SLAP may have other consequences. In addition to negatively regulating cellular signaling, SLAP also interacts with c-Cbl to down-regulate expression of T and B cell receptors (Dragone *et al.*, 2006; Myers *et al.*, 2006) and cortical F-actin assembly in stimulated fibroblasts (Sirvent *et al.*, 2008). Whether the FcεRI signaling complex and F-actin assembly are similarly targeted in a c-Cbl-dependent manner in mast cells will require further study. Our preliminary data (see **Results** section) indeed suggest that SLAP may negatively regulate surface expression of FcεRI at least in resting cells. Although increased expression of FcεRI in siRNA-treated cells might account for the enhancement of signaling events, this is probably unlikely because RBL-2H3 cells express far more receptors than is required for maximal degranulation (Maeyama *et al.*, 1986).

In conclusion, our data indicate that Ag stimulation of RBL-2H3 cells results in substantial upregulation of the inhibitory regulator SLAP which has significant impact on major signaling events and on delayed functional responses such as the production of IL-3 and MCP-1. The upregulation of SLAP thus provides an another mechanism for suppressing mast cell activity especially at late stages of activation in addition to those mediated through Lyn (Xiao *et al.*, 2005), PTEN, SHIP (Furumoto *et al.*, 2006), SHP1, and SHP2 (Kimura *et al.*, 1997a; Kimura *et al.*, 1997b). Also, SLAP appears to have the same profile of actions in RBL-2H3 cells as that previously described for T cells and B cells.

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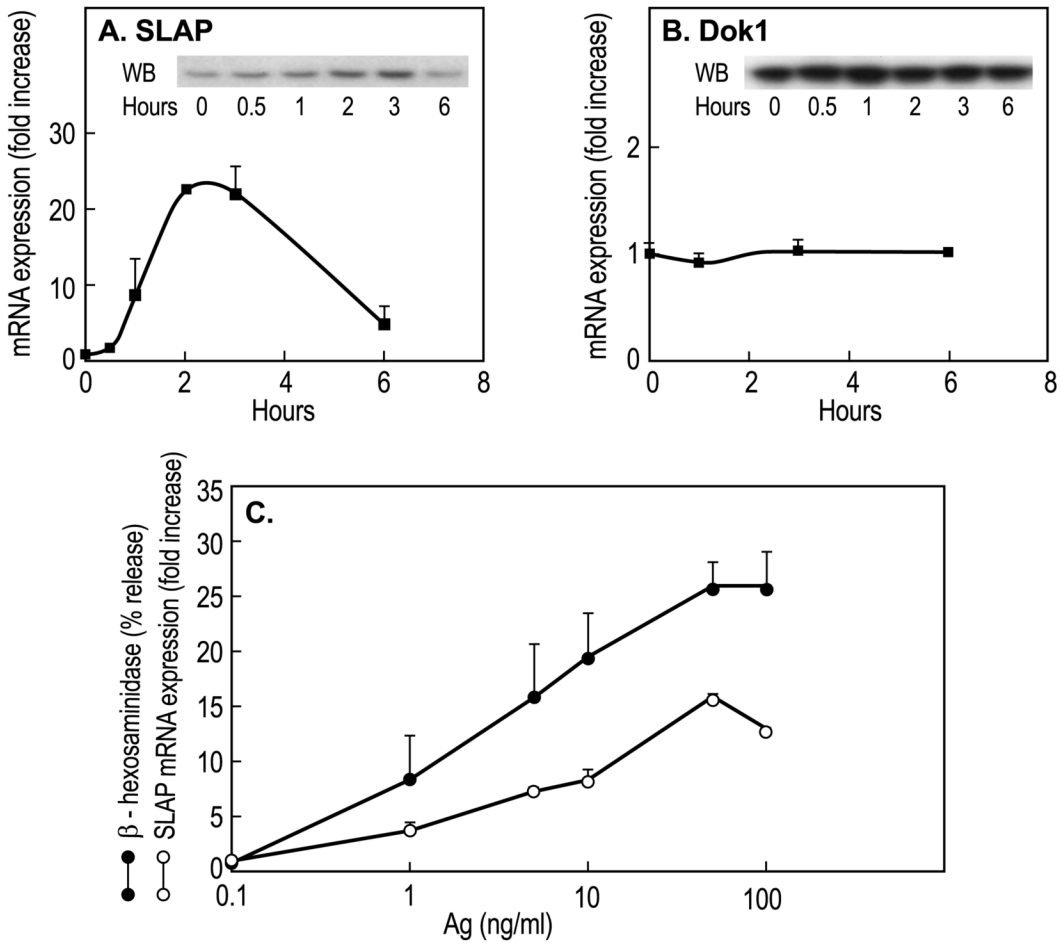

Fig. 1.

Increased expression of SLAP mRNA and protein in response to Ag-stimulation. RBL-2H3 cells sensitized overnight with 50 ng/ml DNP-specifc IgE were stimulated with 50 ng/ml Ag. Panels A and B, cells were stimulated for the indicated times and relative levels of DOK1 and SLAP mRNA and protein (insets) were determined by real-time PCR and immunoblotting respectively. Error bars show Standard Deviations for PCR determinations. The data indicate relative levels of mRNA (zero hours equals 1) and are from one of two similar experiments. Panel C compares the extent of increase of SLAP protein and degranulation with different concentrations of Ag. SLAP was measured 3 h and degranulation 20 min after addition of Ag. Degranulation was assessed by calculating per cent of cellular β-hexosaminidase (a granule marker) that was released into the medium. Data (mean ± SEM) were from four separate cultures and is representative of two experiments.

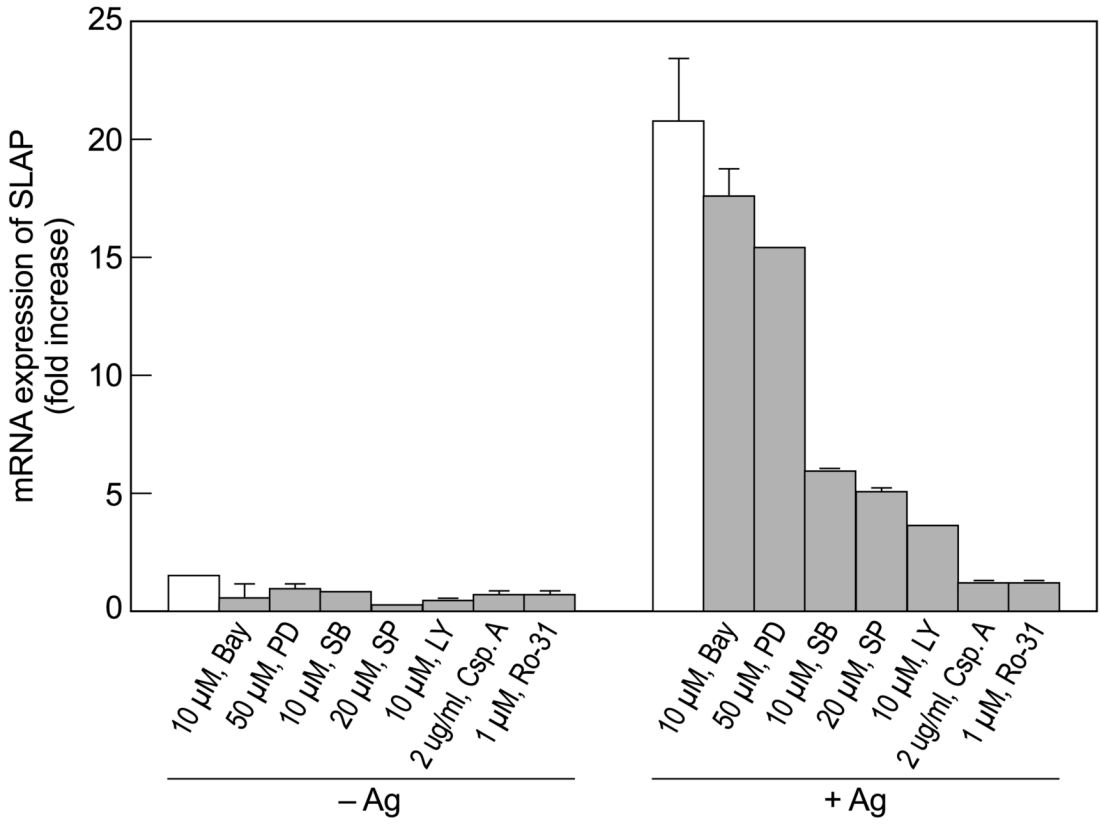

Fig. 2.

Suppression of production of SLAP mRNA by inhibitors of signaling pathways. RBL-2H3 cells were exposed to the indicated concentrations of inhibitors for 20 min before addition of 50 ng/ml Ag. SLAP mRNA was determined 2 h after addition of Ag by real time PCR. Data (mean ± SEM) are from one experiment that is representative of two separate experiments. Abbreviations: Bay, Bay 11–7082; PD, PD-98059; SB, SB 202190; SP, SP 600125; LY, LY 294002; Csp.A, cyclosporine; Ro-31, Ro-31 8220.

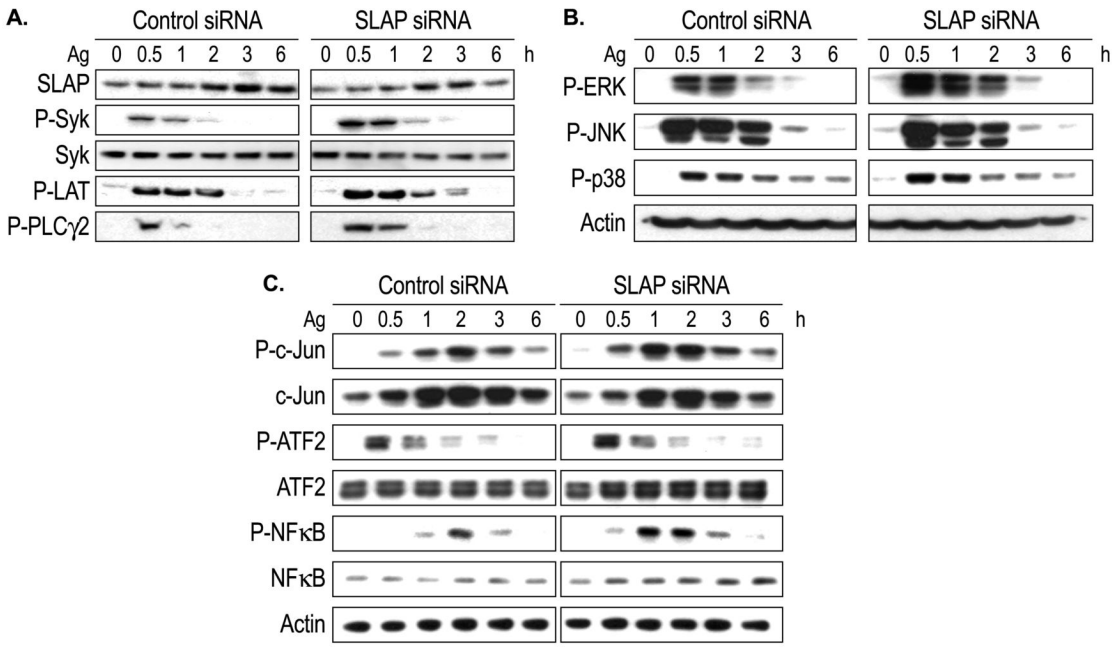

Fig. 3.

Effects of anti-SLAP siRNA on phosphorylation of signaling molecules and transcription factors in Ag-stimulated cells. Control and siRNA-treated RBL-2H3 cells were stimulated with 50 ng/ml Ag for the times shown for determination of the extent of phosphorylation of the indicated proteins as determined by PAGE and immunoblotting (see Materials and Methods). The results shown are typical of three or more experiments.

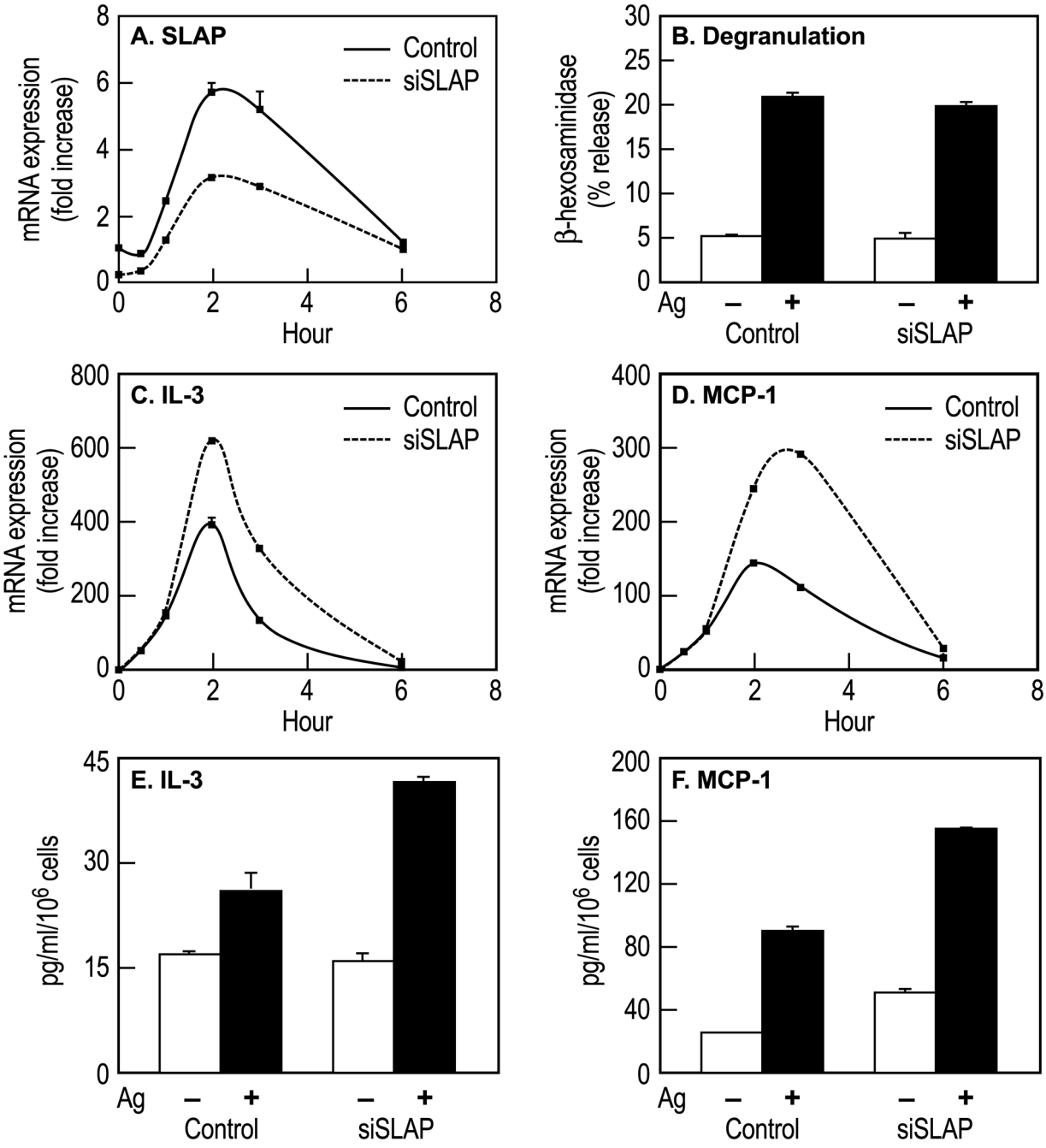

Fig. 4.

Effects of anti-SLAP siRNA on degranulation and production of IL-3 and MCP-1 mRNA and protein. Control and siRNA-treated RBL-2H3 cells were stimulated with 50 ng/ml Ag for the indicated times (Panels A, C–F) or for 20 min (Panel A) for the measurement of SLAP mRNA (Panel A), release of β-hexosaminidase (Panel B), intracellular levels of IL-3 and MCP-1 mRNA (Panels C and D), and IL-3 and MCP-1 released into the medium (Panels E and F). Values in panels A, C, and D indicate fold increase in mRNA relative to the zero time point (equals 1), those in panel B indicate percent release of β-hexosaminidase from cells, and those in panels E and F indicate concentrations of IL-3 or MCP-1 in culture medium. Values are

mean ± SEM of data from three cultures in one experiment that is representative of two or more experiments.