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Cutting Edge: Dexamethasone Negatively Regulates Syk in Mast Cells by Up-Regulating Src-Like Adaptor Protein

Takaaki Hiragun, Ze Peng, and Michael A. Beaven

We have identified Src-like adaptor protein (SLAP) as one of several dexamethasone-inducible inhibitory regulators in mast cells. SLAP is a known inhibitor of T cell signaling and interacts with the tyrosine kinase, Zap70. Exposure of RBL-2H3 mast cells to dexamethasone markedly increased expression of SLAP. Cells so exposed or made to overexpress SLAP exhibited reduced Ag-stimulated phosphorylation of Syk (a cognate of Zap70), linker for activation of T cells, phospholipase C, and ERK. Ca2+ mobilization, Ca2+-dependent degranulation, and ERK-dependent release of arachidonic acid were suppressed as well. Small interfering RNA directed against SLAP blocked the induction of SLAP and reversed the inhibitory effects of dexamethasone on phosphorylation of Syk, linker for activation of T cells, and phospholipase C, but not downstream events, which are likely suppressed by up-regulation of downstream of tyrosine kinase-1 and MAPK phosphatase-1. The induction of these inhibitory regulators may contribute to the immunosuppressive activity of dexamethasone in mast cells.

Gluocorticoids are among the most effective anti-inflammatory agents for the treatment of mast cell-related allergic diseases, but their use is limited by undesirable side effects (1, 2). The glucocorticoids, acting via the glucocorticoid receptor, can positively or negatively regulate gene transcription by mechanisms known as transactivation and transrepression, respectively. The anti-inflammatory actions of glucocorticoids are attributed to the suppression of the transcriptional activity of factors that regulate cytokine gene transcription in inflammatory cells (3, 4). However, glucocorticoids such as dexamethasone also inhibit degranulation, production of inflammatory lipids, and signaling processes that include the Ras/ERK/phospholipase (PL) A2 pathway and PI3K-dependent signaling events that lead to degranulation in mast cells (5–7). These inhibitory actions occur via the glucocorticoid receptor, are of slow onset, and are apparent with as little as 5–10 nM dexamethasone. There are indications that these inhibitory actions may occur through up-regulation of inhibitory factors such as downstream of tyrosine kinase (Dok)-1 (8) and MAPK phosphatase-1 (MKP-1) (9, 10). Dok-1, which negatively regulates Ras by recruiting the Ras GTPase-activating protein, is increased several fold on treatment of RBL-2H3 mast cells with dexamethasone. As a consequence, the entire Ras/Raf1/ERK pathway as well as release of arachidonic acid is suppressed in Ag-stimulated cells (8). MKP-1 is one of a family of dual-specificity protein phosphatases (also known as DUSPs) that dephosphorylate and inactivate ERK and p38 MAPK. Dexamethasone up-regulates expression of MKP-1 in mast cells (10) and other types of cells (11–13) and in this manner negatively regulates ERK.

In a search for other inducible inhibitory regulators in mast cells, we found by microarray analysis that dexamethasone increased expression of RNA transcripts of Src-like adaptor protein (SLAP). Relatively little is known about this protein. It is reported to negatively regulate T cell function by down-regulating expression of TCR (14) and TCR signaling by associating with Zap70 and c-Cbl (15–19). SLAP possesses Src homology (SH)2 and SH3 domains that are homologous to those in Src family kinases (20). As reported in this study, overexpression of SLAP mimics the effects of dexamethasone by suppressing phosphorylation of the tyrosine kinase Syk, which is the Zap70 counterpart in mast cells (21), and downstream events. Suppression of the induction of SLAP by small interfering RNA (siRNA) reverses the inhibitory actions of dexamethasone on phosphorylation of Syk, LAT, and PLCγ, but not on more distal events to indicate that other dexamethasone-inducible inhibitory regulators such as Dok-1 and MPK-1 still operate.

Materials and Methods

Reagents and Abi

The sources of reagents were as follows: dexamethasone, dinitrophenyl (DNP)-human serum albumin, and anti-DNP IgE (Sigma-Aldrich); polyclonal Abs against SLAP (C-19), Syk (C-20), Syk (N-19), and PLCγ2 (Q-20) (Santa Cruz Biotechnology); Abs against phosphotyrosine (4G10) and linker for activation of T cells (LAT) (Upstate Biotechnology); and mAbs against heat shock protein

5 Abbreviations used in this paper: PL, phospholipase; Dok, downstream of tyrosine kinase; MKP-1, MAPK phosphatase-1; SLAP, Src-like adaptor protein; SH, Src homology; siRNA, small interfering RNA; DNP, dinitrophenyl; LAT, linker for activation of T cells.
90 (BD Biosciences Pharmingen). All other Abs, including those against phosphorylated ERK1/2 (Thr202/Tyr204), LAT (Tyr191), and Src (Tyr416), were obtained from Cell Signaling Technology.

**Cell culture, immunoprecipitation, and Western blot analysis**

RBL-2H3 cells were maintained in culture as described before (8) and incubated overnight (18 h) at 37°C with 50 ng/ml anti-DNP-IgE in the presence or absence of dexamethasone unless indicated otherwise. Growth medium was replaced with glucose saline/PiPES buffer (22) and stimulated with 20 ng/ml DNP-human serum albumin for 15 min or for the times indicated. The cultures were placed on ice for subsequent assays.

For immunoprecipitation and Western blot analysis, cells lysates were incubated with the specified Abs and then with protein G-Sepharose 4 Fast Flow beads (Amersham Biosciences) before separation of immunoprecipitated proteins by SDS-PAGE (8). Blots were probed with the indicated primary Abs as described elsewhere (8).

**Microarray analysis and RT-PCR**

Total RNA was extracted from nontreated RBL-2H3 cells and from cells treated with 100 nM dexamethasone for 1, 4, or 12 h by using the RNeasy Mini kit (Qiagen). Total RNA (8 μg) was converted to ss- and then dscDNA by using SuperScript Choice system (Invitrogen Life Technologies) and T7-oligos (IT) promoter primer kit (Alzyme), dscDNA was converted to biotin-labeled antisense cRNA for application to GeneChip arrays (Affymetrix) as described previously (8). For RT-PCR, aliquots of the ssDNA were used for amplification of cDNA fragments of rat SLAP and GAPDH by use of AmpliTaq Gold DNA polymerase (PerkinElmer): sense primer for SLAP, 5′-AGATTGGTAGCTCACCACATACCAGCCGGTGTTTCGTCCTTTCCA-3′; antisense primer for SLAP, 5′-GATTCGTCCACTCTAACAGCTCCGATGGT-3′; sense primer for GAPDH, 5′-TACAGACGCTGCGACCTGCT-3′; antisense primer for GAPDH, 5′-TACAGACGCTGCGACCTGCTGATGGT-3′. PCR fragments were digested with BamHI and XbaI and then ligated into the same sites of pEF6/V5-His vector (Invitrogen Life Technologies).

**SLAP expression vectors**

sscDNA was generated with SuperScript II from 5 μg of total RNA obtained from dexamethasone-treated mouse bone marrow-derived mast cells with the RNeasy Mini Kit (Qiagen). Mouse SLAP (NM_009192) was amplified from dexamethasone-treated mouse bone marrow-derived mast cells with the Cytosolic 5′/H11032; and antisense RNeasy Mini Kit (Qiagen). Mouse SLAP (NM_009192) was amplified from dexamethasone-treated mouse bone marrow-derived mast cells with the Cytosolic 5′/H11032; and antisense RNeasy Mini Kit (Qiagen). Mouse SLAP (NM_009192) was amplified from dexamethasone-treated mouse bone marrow-derived mast cells with the Cytosolic 5′/H11032; and antisense RNeasy Mini Kit (Qiagen). Mouse SLAP (NM_009192) was amplified from dexamethasone-treated mouse bone marrow-derived mast cells with the Cytosolic

**SLAP siRNA expression vector**

siRNA against rat SLAP was generated with the Silencer Express Kit, pSEC neo (Ambion) according to instructions: sense oligonucleotide, 5′-CGGGATCCGGATGGGAATACATGGTAAATCCG-3′; and antisense primer, 5′-CTGCTCGAGTACCAAGCGAATGCTTTCC-3′. The PCR fragments were digested with BamH1 and XbaI and then ligated into the same sites of pEF6/V5-His vector (Invitrogen Life Technologies).

**Transient transfection**

All plasmids were purified by the EndoFree Plasmid Maxi Kit (Qiagen). RBL-2H3 cells were suspended in DMEM containing 25 mM HEPES at a concentration of 4 × 10⁶ cells/ml. Cells (100 μl) were mixed with 15 μg of plasmid and transfected by electroporation (Gene Pulser; Bio-Rad) at 250 V/250 μF. The percentage of transfected cells, as determined by cotransfection with pd2YEFP-N1 (BD Clontech) or pEF6/V5-His-LacZ (Invitrogen Life Technologies), averaged 61 and 42%, respectively. Transfected cells were used within 24 h.

**Measurement of degranulation, release of arachidonic acid, and cytosolic Ca²⁺**

Degranulation and release of [³¹C]arachidonic acid were determined by measurement of release of the granule marker, β-hexosaminidase, and release of [³¹C]arachidonic acid (8, 24). Cytosolic Ca²⁺ was measured in fura-2-loaded cells in a Wallac VICTOR2 plate reader (PerkinElmer Life Sciences) as described elsewhere (6).

**Results and Discussion**

**Dexamethasone increases the expression of SLAP and represses phosphorylation of Syk**

The microarray analysis of RBL-2H3 cells indicated an 11-fold increase in mRNA for SLAP 12 h after the addition of dexamethasone to RBL-2H3 cell cultures (data not shown). Levels of SLAP mRNA, as measured by RT-PCR, reached near maximal levels by 4 h and remained elevated for at least 12 h after the addition of dexamethasone (Fig. 1A). SLAP mRNA could be detected in nontreated cells by increasing the number of PCR cycles (data not shown). SLAP protein was barely detectable before addition of dexamethasone but was readily detected by immunoblotting at 12 h (Fig. 1B). It was also apparent that Ag-induced tyrosine phosphorylation of Syk (Fig. 1C), and its downstream target, the adaptor protein LAT (data not shown), was substantially reduced in dexamethasone-treated cells.

**SLAP associates with and suppresses phosphorylation of Syk**

The possibility that SLAP itself suppresses phosphorylation of Syk was examined in mock and SLAP-transfected RBL-2H3 cells. Syk coimmunoprecipitated with SLAP in mock-transfected cells and to a greater extent in SLAP-transfected cells, and this association was most apparent after Ag stimulation (Fig. 1D). Ag-induced tyrosine phosphorylation of Syk (Fig. 1E) was much reduced in SLAP-transfected cells as compared with mock-transfected cells. In contrast, overexpression of SLAP did not diminish the extent of activating phosphorylations of Src

**FIGURE 1.** Dexamethasone increases expression of SLAP and represses phosphorylation of Syk

A. Dexamethasone increases expression of SLAP. B. IgE-primed RBL-2H3 cells were treated or not with dexamethasone for 18 h and then stimulated with Ag for the indicated times or 12 h for measurement of SLAP mRNA by RT-PCR (A) and SLAP protein by immunoprecipitation and immunoblotting (B). GAPDH or heat shock protein (Hsp) 90 were used as loading controls. C. Cells were treated or not with dexamethasone for 18 h and then stimulated with Ag for the indicated times for immunoprecipitation (IP) of Syk and immunoblotting with anti-phosphotyrosine (anti-PY) and anti-Syk Abs. D and E. Cells were transiently transfected with mock or SLAP and then incubated with Ag-specific IgE overnight. Cells were stimulated or not with Ag for 15 min or as indicated. Immunoblots were prepared from IP SLAP for detection of Syk (D) and from IP Syk for detection of phosphorylated Syk with the anti-phosphotyrosine Ab (anti-PY) and Syk (E). Whole cell lysates (WCL) were also immunoblotted for detection of SLAP. A representative immunoblot of three experiments is shown for each panel.
kinases such as Lyn and Src as determined by immunoprecipitation and blotting with anti-phospho-Src(Y416) (data not shown).

Overexpression of SLAP suppresses phosphorylation of PLC\(\gamma\)2 and ERK and downstream events

Treatment with dexamethasone suppresses activating phosphorylations of PLC\(\gamma\)2 and ERK, the increase in intracellular Ca\(^{2+}\) (calcium signal), Ca\(^{2+}\)-dependent degranulation, and Ca\(^{2+}\)/ERK-dependent generation of arachidonic acid by PLA\(_2\) (5–7). As in dexamethasone-treated cells, in cells made to overexpress SLAP the phosphorylation of LAT (Fig. 2A), PLC\(\gamma\)2 (Fig. 2B), and ERK (Fig. 2C), calcium signal (Fig. 2D), degranulation (Fig. 2E), and release of arachidonic acid (Fig. 2F) were substantially reduced as compared with mock-transfected cells. In concurrent experiments, comparable decreases in calcium signal (Fig. 2E), degranulation, and release of arachidonic acid (data not shown, but see Ref. 6) were observed in cells treated with 20 nM dexamethasone. These results indicated that overexpressed SLAP mimicked the inhibitory effects of dexamethasone.

The effects of siRNA directed against SLAP

To verify that SLAP negatively regulates Syk and Syk-dependent pathways, cells were made to transiently express siRNA against SLAP. After treatment with dexamethasone, such cells exhibited much reduced expression of SLAP (Fig. 3A) and complete reversal of the inhibitory effects of dexamethasone on tyrosine phosphorylation of Syk (Fig. 3B), LAT (Fig. 3C), and PLC\(\gamma\) (Fig. 3D), and a partial reversal of the inhibitory effects on the calcium signal (compare Fig. 3E with 3F) in response to Ag stimulation as compared with cells that were transfected with a negative control. Nevertheless, expression of the siRNA failed to reverse the inhibitory actions of dexamethasone on phosphorylation of ERK (data not shown), degranulation (Fig. 3G), and release of arachidonic acid (Fig. 3H). Our presumption is that up-regulation of Dok-1 and MPK-1 by dexamethasone

FIGURE 2. Overexpression of SLAP suppresses phosphorylation of LAT, PLC\(\gamma\)2, and ERK, calcium signal, degranulation, and release of [\(^{14}\)C]arachidonic acid. RBL-2H3 cells, transfected with vector alone (mock) or SLAP, were incubated with IgE overnight and then stimulated with Ag for 15 min or as indicated. A–C, Immunoblots were prepared from whole cell lysates (A and C) and immunoprecipitated PLC\(\gamma\)2 (B). The immunoblots were probed with Abs against LAT, PLC\(\gamma\)2, and ERK1/2 as well as their phosphorylated counterparts, LAT(Y191), phosphotyrosine (anti-pY), and phosphorylated (T202/Y204) ERK1/2 (anti-p-ERK) as indicated. D and E, Fura-2-loaded cells were monitored for changes in cytosolic Ca\(^{2+}\) by measurement of the ratio in fluorescence (510 nm) when irradiated at 340 and 380 nm. The data are from mock- and SLAP-transfected cells (D) and normal RBL-2H3 cells after treatment with vehicle or 100 nM dexamethasone (Dex) for 18 h (E). F and G, Release of the granule marker, \(\beta\)-hexosaminidase (F), and [\(^{14}\)C]arachidonic acid from [\(^{14}\)C]arachidonate-labeled cells (G): values are percentage of total \(\beta\)-hexosaminidase or \(^{14}\)C label released into the medium. Data are representative examples (A–E), or the mean \(\pm\) SEM of values (F and G), from three experiments. Significant differences (paired \(t\) test) between SLAP- and mock-transfected cells are shown; *, \(p < 0.05\) and **, \(p < 0.01\).

FIGURE 3. Anti-SLAP siRNA reverses some but not all inhibitory actions of dexamethasone. RBL-2H3 cells, transfected with negative control siRNA (NC-siRNA) or anti-SLAP siRNA (SLAP-siRNA), were incubated with IgE, with or without 100 nM dexamethasone, for 18 h. Cells were stimulated with Ag for 15 min or as indicated. A–D, Immunoblots were prepared from immunoprecipitates (IP) or whole cell lysates (WCL) for detection of SLAP (A), Syk (B), LAT (C), PLC\(\gamma\)2 (D), and their phosphorylated counterparts exactly as described in previous legends. E–H, Changes in cytosolic Ca\(^{2+}\) (E and F), degranulation (G), and release of [\(^{14}\)C]arachidonic acid (H) were also monitored as described for Fig. 2. Data are representative examples, or the mean \(\pm\) SEM (G and H), from three experiments.
would account for this inability to reverse the inhibition of phosphorylation of ERK and release of arachidonic acid. In addition, Dok-1 can inhibit the calcium signal and degranulation in human mast cells by interacting with the SHIP (25).

Concluding comments

The identification of SLAP along with Dok-1 and MPK-1 as dexamethasone-regulated molecules makes a compelling case that glucocorticoids act by induction of inhibitory signaling factors through gene activation (transactivation) in addition to the inhibition of cytokine gene transcription (transrepression). Therefore, glucocorticoids that are designed for selectivity toward transrepression to minimize metabolic side effects, sometimes referred to as dissociated glucocorticoids (2), may lose an important component of therapeutic activity. The present studies also identify SLAP as a previously unrecognized regulator of Syk activity in mast cells.

Disclosures

The authors have no financial conflict of interest.

References