Inhibition by dexamethasone of interleukin 13 production via glucocorticoid receptor-mediated inhibition of c-Jun phosphorylation

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Abstract The antigen stimulation of RBL-2H3 cells induced interleukin 13 (IL-13) production, which was inhibited by the steroidal anti-inflammatory drug dexamethasone and by the c-Jun N-terminal kinase (JNK) inhibitor SP600125. Dexamethasone did not inhibit the antigen-induced phosphorylation of JNK but inhibited that of c-Jun. In a cell-free system, the phosphorylation of glutathione *S*-transferase-fused c-Jun by recombinant JNK was not inhibited by dexamethasone but was inhibited by the addition of recombinant glucocorticoid receptor (GR). These findings suggest that the inhibition of antigen-induced IL-13 production by dexamethasone is due to the GR-mediated inhibition of c-Jun phosphorylation induced by JNK.

Key words: Dexamethasone; c-Jun N-terminal kinase; c-Jun; Glucocorticoid receptor; Interleukin 13; RBL-2H3 cell line

1. Introduction

Glucocorticoids are widely used for the treatment of inflammatory diseases such as asthma, rheumatoid arthritis and autoimmune diseases. The most striking effect of glucocorticoids is the inhibition of the expression of inflammatory gene products, which include cytokines, enzymes and adhesion molecules [1]. Interleukin 13 (IL-13) is one of the cytokines produced by mast cells and plays critical roles in remodeling of the airway in an experimental model of asthma [2]. The inhibition by glucocorticoids of IL-13 production in mast cells [3] is considered critical to the anti-asthmatic effects of glucocorticoids on mast cells although the inhibitory mechanism has not been fully clarified. The IL-13 promoter contains the binding sites for the transcription factors GATA-3, nuclear factor of activated T cells and activating protein-1 (AP-1) [4]. Glucocorticoids bind to the glucocorticoid receptor (GR) and modulate gene expression either by direct binding of the glucocorticoid–GR complex to the glucocorticoid-responsive element or indirectly by inhibiting the activities of other transcription factors such as AP-1 [1].

A major component of AP-1 is a heterodimer of c-Jun and c-Fos. The phosphorylation of c-Jun at Ser^{63} and Ser^{73} by c-Jun N-terminal kinase (JNK) enhances AP-1 activity [5]. Several mechanisms by which glucocorticoids inhibit AP-1 activity are proposed: (1) inhibition of the binding of AP-1 to DNA by direct binding of GR to AP-1 [6–8]; (2) competitive reduction of the interaction between AP-1 and the transcriptional coactivator CREB binding protein (CBP) or p300 via the interaction of GR with CBP/p300 [9]; (3) suppression of the activation of JNK [10–12]; and (4) induction of the inhibitory protein, glucocorticoid-induced leucine zipper [13]. However, there are several reports opposing these hypotheses [14–17]. In this study, we examined the effect of dexamethasone on the activation of AP-1 in connection with the inhibition of IL-13 production in the rat basophilic leukemia cell line RBL-2H3. We found that dexamethasone inhibits the antigen-induced phosphorylation of c-Jun without inhibiting the activation of JNK, and proposed a novel mechanism by which dexamethasone inhibits AP-1 activation in RBL-2H3 cells.

2. Materials and methods

2.1. Cell culture

Rat basophilic leukemia RBL-2H3 cells (Health Science Research Resources Bank, Osaka, Japan) (2×10^5 cells/well in a 24-well cluster dish, or 1×10^6 cells/well in a 6-well cluster dish) were incubated overnight in Eagle's minimal essential medium (EMEM) containing 10% (v/v) fetal bovine serum (FBS, Sigma Chemical, St. Louis, MO, USA), and 0.1% (v/v) conditioned medium of dinitrophenol (DNP)-specific IgE-producing hybridoma (kindly supplied by Dr. Kazutaka Maeyama, Ehime University, Ehime, Japan).

2.2. Drug treatment

Dexamethasone (Sigma), the JNK inhibitor SP600125 (Biomol, Plymouth Meeting, PA, USA), the p38 mitogen-activated protein (MAP) kinase inhibitor SB203580 (Calbiochem-Novabiochem, San Diego, CA, USA) and the MAP extracellular signal-regulated kinase kinase (MEK) inhibitor PD98059 (New England Biolabs, Beverly, MA, USA) dissolved in dimethylsulfoxide (DMSO) were diluted with EMEM. After overnight incubation of the cells (2×10^5 cells) in the presence of DNP-specific IgE, the medium was changed to 0.4 ml of EMEM containing 5% (v/v) FBS in the presence or absence of the antigen DNP-conjugated human serum albumin (HSA) (Sigma Chemical) (50 ng/ml), dexamethasone and/or the JNK inhibitor SP600125 at the concentration indicated and the cells were then incubated for specific periods. For the pretreatment of the cells with SB203580 and PD98059, the cells were incubated in 0.36 ml of the medium containing SB203580 (10 μM) or PD98059 (10 μM) for 10 min. The cells were then stimulated by the addition of 0.04 ml of the medium containing the antigen (500 ng/ml) and 10 μM of the

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Abbreviations: AP-1, activating protein 1; BSA, bovine serum albumin; CBP, CREB binding protein; DMSO, dimethylsulfoxide; DNP, dinitrophenyl; EMEM, Eagle's minimal essential medium; FBS, fetal bovine serum; GR, glucocorticoid receptor; GST, glutathione *S*-transferase; HSA, human serum albumin; IL-13, interleukin 13; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MEK, MAP extracellular signal regulated kinase kinase

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corresponding inhibitor into each well, and further incubated for 4 h. The final concentration of DMSO was adjusted to be 0.2% (v/v).

2.3. Measurement of IL-13

After the antigen stimulation, the conditioned medium was collected and centrifuged at 450×*g* and 37°C for 5 min. The IL-13 level in the supernatant fraction was determined using a rat IL-13 ELISA kit (BioSource, Camarillo, CA, USA).

2.4. Immunoblotting

After the antigen stimulation, the cells (5×10^6 cells/well in a 6-well cluster dish) were lysed in 150 μl of a lysis buffer (20 mM HEPES, pH 7.3, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM EDTA, 50 mM NaF, 2.5 mM *p*-nitrophenylphosphate, 10 μg/ml phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 and 5 μg/ml leupeptin). The cell lysate was then sonicated for 1 min using a Handy Sonic Disruptor (Model UR-20P, Tomy Seiko, Tokyo, Japan). Proteins in the cell lysate were separated by electrophoresis on sodium dodecyl sulfate–polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The levels of c-Jun, phospho-c-Jun, JNK, phospho-JNK and actin were determined by immunoblotting using polyclonal antibodies against the epitope corresponding to amino acids 1–79 mapping at the amino-terminus of c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA, USA), c-Jun phosphorylated at Ser^{73} (New England Biolabs), full-length JNK2 (Santa Cruz Biotechnology), JNK phosphorylated at Thr^{183} and Tyr^{185} (Promega, Madison, WI, USA), and the epitope mapping at the carboxy-terminus of actin (Santa Cruz Biotechnology), respectively.

2.5. Preparation of glutathione S-transferase (GST)-fused c-Jun

The rat c-Jun cDNA was amplified by polymerase chain reaction (PCR) using pBSrc-Jun-1 (Riken Gene Bank, Tsukuba, Japan) as a template and the following primers, 5′-GGATCCATGACTGCAAAGATGGAAA-3′ (sense) and 5′-GAATTCTCAAAACGTTTGCAACT-3′ (antisense), containing a *Bam*HI and *Eco*RI overhang, respectively. The PCR products were digested with *Bam*HI and *Eco*RI, and inserted into pGEX4T (Amersham Pharmacia Biotech, Uppsala, Sweden). pGEX4T-c-Jun was introduced into *Escherichia coli* DH5α and recombinant GST-c-Jun was expressed by incubation for 4 h at 25°C in the presence of 0.5 mM of isopropyl-β-D-galactopyranoside (Takara, Kyoto, Japan). The recombinant GST-c-Jun was purified using glutathione-Sepharose beads (Amersham Pharmacia Biotech).

2.6. Analysis of JNK activity

GST-c-Jun-immobilized glutathione-Sepharose beads (50% (v/v) gel, 20 μl) and JNK (0.25 pmol, Upstate Biotechnology, Lake Placid, NY, USA) were incubated for 20 min at 37°C in the presence or absence of GR (0.05–0.25 pmol, Panvera, Madison, WI, USA), bovine serum albumin (BSA, fraction V, 10 nmol, Wako Pure Chemicals, Tokyo, Japan) and dexamethasone (100 nM, Sigma) in 50 μl of assay buffer (20 mM HEPES, pH 7.8, 20 mM $MgCl_2$ and 20 mM β-glycerophosphate) containing 10 mM ATP (Sigma). After the incubation, GST-c-Jun-immobilized glutathione-Sepharose beads were washed with 0.5 ml of phosphate-buffered saline (pH 7.4) three times. GST-c-Jun was eluted with sample buffer (50 mM Tris–HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate, 850 mM 2-mercaptoethanol and 10% (v/v) glycerol) and the phosphorylation of GST-c-Jun was determined by immunoblotting as described above.

2.7. Statistical analysis

Results were analyzed for statistical significance using Student's *t*-test for unpaired observations.

3. Results

3.1. Effects of dexamethasone on the antigen-induced IL-13 production

The antigen stimulation of IgE-sensitized RBL-2H3 cells induced IL-13 production at 4 h (Fig. 1A). The simultaneous addition of dexamethasone (1, 10, and 100 nM) with the antigen decreased the IL-13 level in the conditioned medium at 4 h in a concentration-dependent manner (Fig. 1A). The inhibition by dexamethasone was observed from 1 to 4 h (Fig. 1B).

3.2. Effects of MAP kinase inhibitors on the antigen-induced c-Jun phosphorylation and IL-13 production

To clarify the involvement of JNK in the antigen-induced IL-13 production, we examined the effect of the JNK inhibitor SP600125. Stimulation with the antigen for 1 h increased both the phosphorylation and the protein production of c-Jun, which were inhibited by SP600125 (20 and 40 μM) in a concentration-dependent manner (Fig. 2A). Equal loading of proteins on all lanes was confirmed by blotting with anti-actin antibody. In the same concentration range, SP600125 inhibited the antigen-induced IL-13 production at 4 h (Fig. 2B). In contrast, the p38 MAP kinase inhibitor SB203580 (10 μM) and the MEK inhibitor PD98059 (10 μM) did not inhibit the antigen-induced production of IL-13 (Fig. 2C).

3.3. Effects of dexamethasone on the antigen-induced phosphorylation of c-Jun and JNK and their protein levels

The antigen-induced increase in the phosphorylation and the protein level of c-Jun reached a maximum 60 min after the antigen stimulation (Fig. 3). Simultaneous treatment with dexamethasone (100 nM) and the antigen decreased both the antigen-induced increase in the levels of c-Jun phosphoryla-

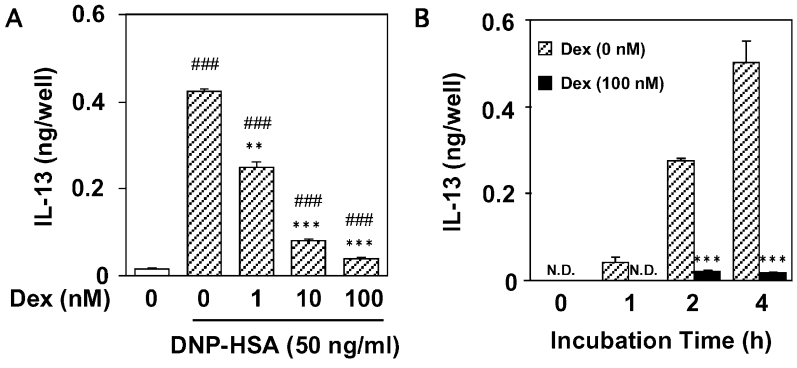

Fig. 1. Effects of dexamethasone on the antigen-induced IL-13 production. IgE-sensitized RBL-2H3 cells (2×10^5 cells) were stimulated with DNP-HSA (50 ng/ml) in 0.4 ml of medium in the presence of the indicated concentrations of dexamethasone (Dex) for 4 h (A) or for the periods indicated (B). The IL-13 concentration in the conditioned medium was determined by ELISA. Vertical bars represent S.E.M. from three wells. Statistical significance: $^{**}P<0.01$ and $^{***}P<0.001$ vs. DNP-HSA alone, $^{###}P<0.001$ vs. non-stimulated control. N.D., not detectable (<1.5 pg/ml).

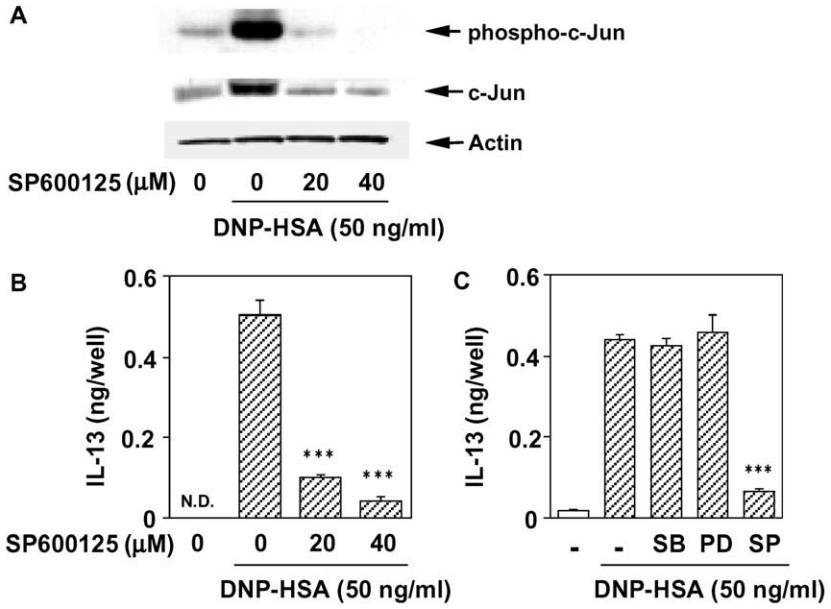

Fig. 2. Effects of SP600125 on the antigen-induced c-Jun phosphorylation and IL-13 production. A: IgE-sensitized RBL-2H3 cells (1×10^6 cells) were stimulated with DNP-HSA (50 ng/ml) in 2 ml of medium in the presence of the indicated concentrations of SP600125 for 1 h. c-Jun, phospho-c-Jun and actin in the cell lysate were detected by immunoblotting. B: IgE-sensitized RBL-2H3 cells (2×10^5 cells) were stimulated with DNP-HSA (50 ng/ml) in 0.4 ml of medium in the presence of the indicated concentrations of SP600125 for 4 h. The IL-13 concentration in the conditioned medium was determined by ELISA. Vertical bars represent S.E.M. from three wells. Statistical significance: $^{***}P<0.001$ vs. DNP-HSA alone. N.D., not detectable (< 1.5 pg/ml). C: IgE-sensitized RBL-2H3 cells (2×10^5 cells) were preincubated for 10 min in 0.36 ml of medium in the presence or absence of SB203580 (SB, 10 μM) or PD98059 (PD, 10 μM). The cells were then stimulated with DNP-HSA (50 ng/ml) for 4 h in 0.4 ml of medium in the presence of the corresponding drugs or SP600125 (SP, 40 μM). The IL-13 concentration in the conditioned medium was determined by ELISA. Vertical bars represent S.E.M. from three wells. Statistical significance: $^{***}P<0.001$ vs. DNP-HSA alone.

tion and c-Jun protein (Fig. 3), but did not affect the antigen-induced increases in the phosphorylation of JNK 1 and JNK 2, which attained a maximum 30 min after the stimulation (Fig. 4). The protein levels of each JNK did not change on treatment with the antigen in the presence or absence of dexamethasone (Fig. 4).

3.4. *Effects of GR on JNK-induced phosphorylation of GST-c-Jun*

Incubation of GST-c-Jun-immobilized glutathione-Sepharose beads with recombinant JNK (rJNK, 0.25 pmol) in the assay buffer containing 10 mM ATP induced the phosphorylation of GST-c-Jun (Fig. 5A). The phosphorylation of GST-

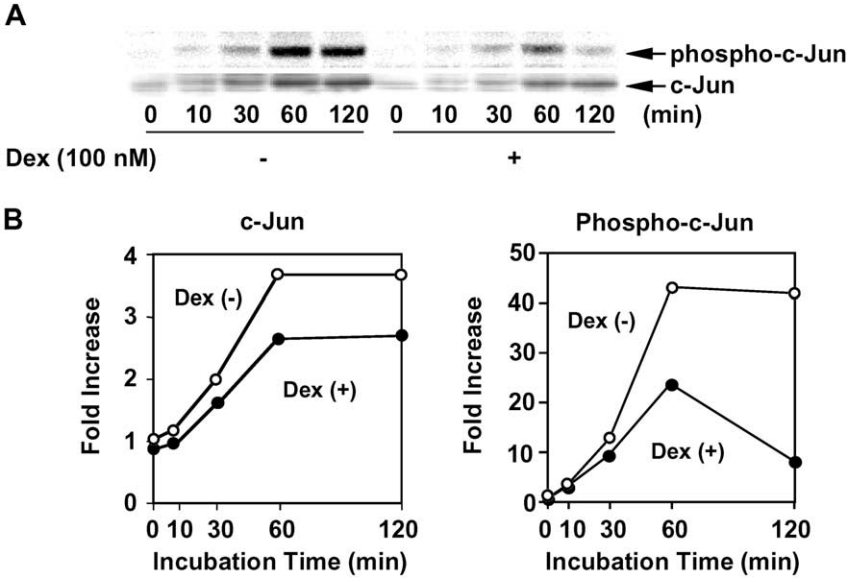

Fig. 3. Inhibition of the antigen-induced phosphorylation and expression of c-Jun by dexamethasone. IgE-sensitized RBL-2H3 cells (1×10^6 cells) were stimulated with DNP-HSA (50 ng/ml) in 2 ml of medium in the presence (+) or absence (−) of dexamethasone (Dex, 100 nM) for the periods indicated. c-Jun and phospho-c-Jun were detected by immunoblotting (A). The levels of c-Jun and phospho-c-Jun were determined by densitometric analysis (B). The density at time 0 in Dex (−) is set to 1.0.

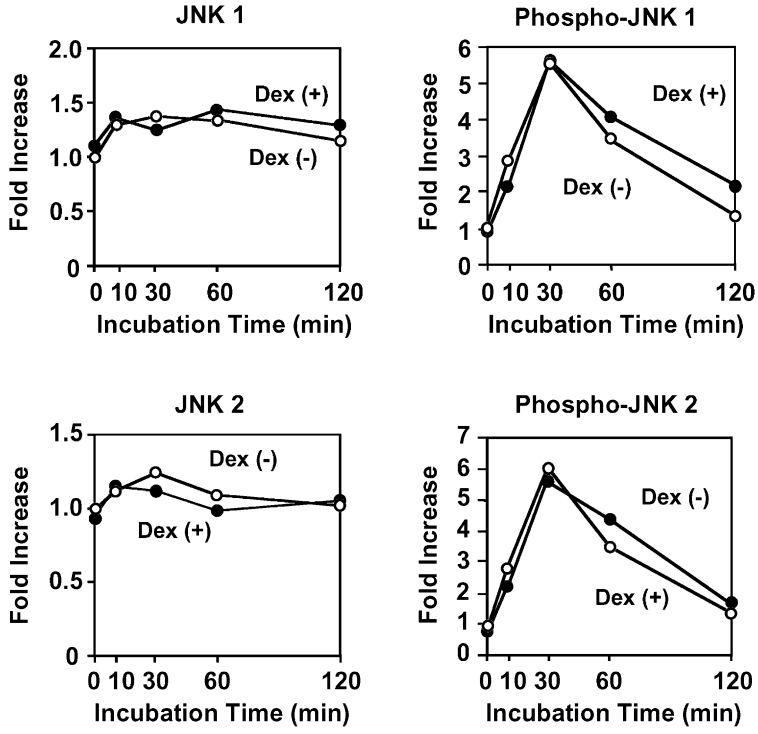

Fig. 4. Effects of dexamethasone on the antigen-induced phosphorylation of JNK. IgE-sensitized RBL-2H3 cells (1×10^6 cells) were stimulated with DNP-HSA (50 ng/ml) in 2 ml of medium in the presence (+) or absence (−) of dexamethasone (Dex, 100 nM) for the periods indicated. JNK 1, JNK 2, phospho-JNK 1 and phospho-JNK 2 were detected by immunoblotting. The levels of JNK 1, JNK 2, phospho-JNK 1 and phospho-JNK 2 were determined by densitometric analysis. The density at time 0 in Dex (−) is set to 1.0.

c-Jun by rJNK was not inhibited by dexamethasone (100 nM) but was inhibited by recombinant GR (rGR, 0.25 pmol) in the presence or absence of the ligand dexamethasone (Fig. 5A). The addition of BSA instead of rGR did not affect the JNK activity (Fig. 5A). rGR inhibited the rJNK-induced phosphorylation of GST-c-Jun in a concentration-dependent manner, and at 0.25 pmol, the same concentration as JNK, the strongest inhibitory effect was observed (Fig. 5B).

4. Discussion

Reflecting that the IL-13 promoter region contains the AP-1 binding site [4], the antigen-induced IL-13 production in RBL-2H3 cells was highly dependent on the phosphorylation of c-Jun, a component of AP-1, by JNK (Fig. 2B), but not regulated by p44/42 ERK MAP kinase and p38 MAP kinase (Fig. 2C). Dexamethasone reduced the antigen-induced phos-

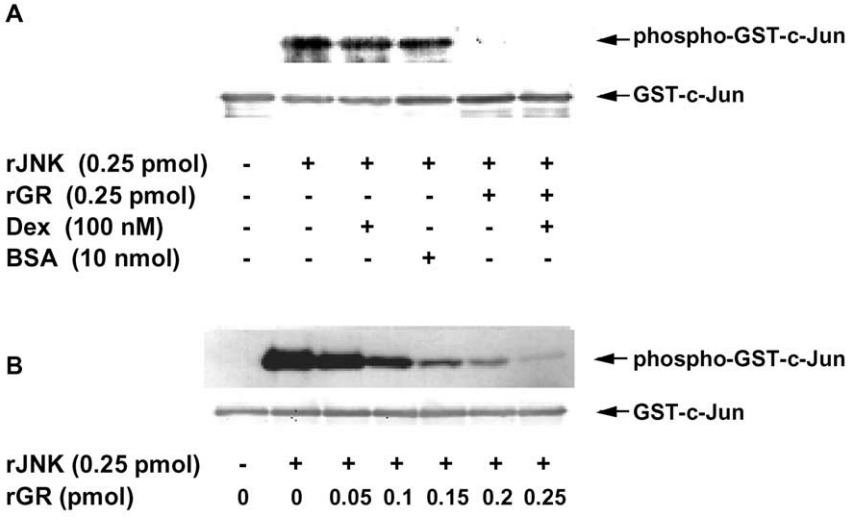

Fig. 5. Inhibition by rGR of rJNK-induced phosphorylation of GST-c-Jun. A,B: GST-c-Jun-immobilized glutathione-Sepharose beads and rJNK (0.25 pmol) were incubated in 50 μl of the assay buffer containing 10 mM ATP for 20 min at 30°C in the presence (+) or absence (−) of BSA (10 nmol), dexamethasone (Dex, 100 nM) and/or rGR (0.25 pmol (A) or the amount indicated (B)). GST-c-Jun and phospho-GST-c-Jun were detected by immunoblotting.

phorylation of c-Jun (Fig. 3), suggesting that the inhibition of the antigen-induced IL-13 production by dexamethasone is mediated by the inhibition of the antigen-induced activation of AP-1. Furthermore, the antigen-induced increase in the c-Jun protein level was suppressed by SP600125 (Fig. 2A) and dexamethasone (Fig. 3), indicating that the expression of c-Jun is also regulated by the phosphorylation of c-Jun.

We reported that dexamethasone inhibited the antigen-induced activation of p44/42 MAP kinase [18] and JNK [12] in RBL-2H3 cells, but in both cases, preincubation with dexamethasone for 6 h or more was required. In addition, the inhibition by dexamethasone of the antigen-induced degranulation and arachidonic acid release in RBL-2H3 cells also required preincubation with dexamethasone for several hours [12]. However, in this study, we demonstrated that dexamethasone inhibited the antigen-induced production of IL-13 without preincubation. We also found that simultaneous treatment with dexamethasone inhibited the antigen-induced phosphorylation of c-Jun without inhibiting that of JNK, i.e. the activation of JNK. Similar results were also observed in the murine macrophage cell line RAW 264.7. Namely, when RAW 264.7 cells were stimulated by lipopolysaccharide (1 μg/ml) in the presence of dexamethasone (100 nM), the lipopolysaccharide-induced phosphorylation of c-Jun was reduced by dexamethasone at 1 h, but that of JNK at 20 min was not inhibited (data not shown). Because the antigen-induced translocation of JNK to the nucleus is not inhibited by dexamethasone [19], it is likely that dexamethasone inhibits c-Jun phosphorylation via inhibition of JNK activity. In a cell-free system using recombinant proteins, we disclosed that the phosphorylation of GST-c-Jun by rJNK was not inhibited by dexamethasone but was inhibited by rGR (Fig. 5A). Furthermore, dexamethasone, the ligand of GR, was not necessary for the rGR-mediated inhibition of JNK activity (Fig. 5A). These findings suggested that GR was translocated into the nucleus by dexamethasone and interfered with the association of activated JNK with c-Jun.

It is reported that GR binds to c-Jun thereby preventing c-Jun from binding to DNA [6–8] and/or a coactivator such as p300/CBP [9]. Taken together, we hypothesized that GR disrupts the binding of JNK to c-Jun to inhibit the phosphorylation of c-Jun by JNK. In addition, because GR is phosphorylated by JNK [20], the interaction between GR and JNK might be involved in the inhibition of the phosphorylation of c-Jun. The molecular mechanisms involved are under investigation in our laboratory.

In conclusion, dexamethasone inhibited AP-1-mediated IL-13 production through GR-mediated inhibition of c-Jun phosphorylation. The inhibition by GR of the phosphorylation of c-Jun by JNK is a novel mechanism by which glucocorticoids inhibit the activation of AP-1.

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