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LIF- and IL-6-Induced Acetylation of STAT3 at Lys-685 through PI3K/Akt Activation

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Signal transducer and activator of transcription 3 (STAT3), which mediates biological actions in many physiological processes, is activated by cytokines and growth factors via specific tyrosine or serine phosphorylation, dimerization and nuclear translocation. A recent study has demonstrated, by using antibody to acetylated lysine, and a STAT3 mutant with Lys-685-to-Arg substitution, that STAT3 is acetylated at Lys-685 by histone acetyltransferase p300, and that acetylation at Lys-685 is critical for STAT3 activation. In the present study, we created an acetyl-specific antibody against STAT3 acetylated at Lys-685, and found that leukemia inhibitory factor (LIF) or interleukin (IL)-6 induced acetylation of STAT3 at Lys-685 in 293T and Hep3B cells. Moreover, acetylation of STAT3 at Lys-685 was suppressed by PI3K inhibitor LY294002, or a dominant negative Akt. Taken together, our findings demonstrate that endogenous STAT3 is acetylated at Lys-685 by LIF or IL-6 through PI3K/Akt activation.

Key words signal transducer and activator of transcription 3; interleukin-6; acetylation; leukemia inhibitory factor; PI3K; Akt

Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT protein family, and is mainly activated by the interleukin (IL)-6 family of cytokines, interferons, epidermal growth factor and leptin.^{1,2} Similar to other members of the STAT family, upon cytokine stimulation, STAT3 is tyrosine-phosphorylated at Tyr-705 by Janus kinases (Jaks), and is subsequently dimerized and translocated to the nucleus to activate its target genes.^{3,4} STAT3 is also phosphorylated at Ser-727 in response to cytokine stimulation.⁵ Phosphorylation of STAT3 at Ser-727 is required for the maximal transcriptional activation of STAT3.⁶ Ser-727 phosphorylation is also shown to increase STAT3 activity through association with other cofactors, such as p300.⁷ Recently, it has also been demonstrated that STAT3 is acetylated by oncostatin M (OSM) or interferon- α (IFN- α) stimulation.⁸ Furthermore, experiments using antibody to acetylated lysine, and a STAT3 mutant with a Lys-685-to-Arg substitution, have revealed that STAT3 is acetylated at Lys-685 by histone acetyltransferase p300, and its acetylation is critical for the formation of stable dimers required for transcriptional activation.

In the present study, we created an acetyl-specific antibody against STAT3 acetylated at Lys-685, and found that leukemia inhibitory factor (LIF) and IL-6 induced acetylation of STAT3 at Lys-685. Furthermore, IL-6-induced acetylation at Lys-685 was suppressed by PI3K inhibitor, LY294002. Moreover, a dominant negative Akt inhibited acetylation of STAT3 at Lys-685 by p300. These results suggest that LIF or IL-6 mediates acetylation of STAT3 at Lys-685 through activation of the PI3K/Akt signaling pathway.

MATERIALS AND METHODS

Reagents and Antibodies Recombinant human LIF was purchased from INTERGEN (Purchase, NY, U.S.A.). Recombinant human IL-6 was kindly provided from Ajinomoto (Tokyo, Japan). PD98059 and LY294002 were purchased

from Calbiochem (La Jolla, CA, U.S.A.). Expression vectors, STAT3, p300, histone deacetylase (HDAC)3, Akt KN, Akt Δ PH and STAT3-LUC were kindly provided by Dr. T. Hirano (Osaka Univ., Osaka, Japan), Dr. T. Taga (Inst. Mol. Embryol. Genet., Kumamoto Univ., Kumamoto, Japan), Dr. E. Seto (H. Lee Moffitt Cancer Ctr. & Res. Inst., FL, U.S.A.), Dr. H. Shibuya (Med. Res. Inst. & Sch. Biomed. Sci., Tokyo Med. Dent. Univ., Tokyo, Japan). Expression vector for STAT3 K685R (STAT3KR) mutant was generated by PCR methods and sequenced (primer sequences are available upon request).⁸ Anti-Myc and anti-STAT3 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-FLAG mAb (M2) and anti-hemagglutinin (HA) antibodies were purchased from Sigma-Aldrich (Saint Louis, MO, U.S.A.). Anti-phospho-STAT3 Tyr705 (pSTAT3 Tyr705), anti-phospho-STAT3 Ser727 (pSTAT3 Ser727), anti-phospho-extracellular signal-regulated kinase (pERK), anti-phospho-Akt (pAkt) and anti-Akt antibodies were purchased from Cell Signaling Technologies (Beverly, MA, U.S.A.). The acetylpeptide used as immunogen for anti-acetyl STAT3 Lys-685 (anti-Ac STAT3 Lys685) was Acetyl-KYCRPESQEHPEAD, corresponding to residues 685 to 698 of human STAT3. The phospho-peptide was conjugated to keyhole limpet hemocyanin as an antigen. Rabbits were immunized and boosted three times at monthly intervals with the conjugate, before blood was collected and immune serum was obtained.

Cell Culture, Transfection and Luciferase Assays Human cervix carcinoma cell line, HeLa and human hepatoma cell line, Hep3B, were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS). Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FCS and transfected by the standard calcium precipitation protocol. Luciferase assay was performed as described.⁹ At 36 h after transfection, the cells were stimulated with LIF (50 ng/ml) for additional 8 h and lysed in 50 μ l of Reporter Lysis Buffer

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(Promega, Madison, WI, U.S.A.) and assayed for luciferase activities according to the manufacturer's instructions. Luciferase activities were normalized to the β -galactosidase activities. Three or more independent experiments were carried out for each assay.

Immunoprecipitation and Immunoblotting The immunoprecipitation and Western blotting assays were performed as described previously.¹⁰ Cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 10 μ g/ml each of aprotinin, pepstatin and leupeptin). An aliquot of total cell lysates or the immunoprecipitate with anti-STAT3 antibody was resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) transfer membrane (PerkinElmer; Boston, MA, U.S.A.). The filters were then immunoblotted with the respective antibody.

RESULTS AND DISCUSSION

Involvement of Lys-685 in STAT3 Activation and Detection of Its Acetylation State by an Acetyl-Specific Antibody

A recent study has demonstrated that STAT3 is acetylated by OSM or IFN- α stimulation, and is acetylated at Lys-685 by histone acetyltransferase p300. Furthermore, acetylation of STAT3 at Lys-685 is critical for the formation of stable dimers required for transcriptional activation.⁸ However, the molecular mechanism of acetylation of STAT3 at Lys-685 upon cytokine stimulation is still unknown. To confirm the role of acetylation of STAT3, we first examined the effect of substitution of Lys-685 to Arg in STAT3 (STAT3K685R) on LIF-induced STAT3 activation, using a STAT3-LUC reporter assay. We then transfected wild-type STAT3 (STAT3 WT), dominant negative STAT3 (STAT3 YF) or STAT3 K685R (KR) together with STAT3-LUC into 293T cells. Dominant negative STAT3 YF has a substitution of Tyr-705 to Phe and has lost its transcriptional activation. After 36 h, the cells were treated with LIF for 8 h, and the STAT3-LUC

activity was determined. As shown in Fig. 1A, STAT3 WT showed marked LIF-induced STAT3-LUC activation. Importantly, STAT3 KR transfectants showed a decrease of LIF-induced STAT3-LUC activation compared to STAT3 WT transfectants. These results indicate that Lys-685 plays an important role in the STAT3 transcriptional activity, and allowed us to monitor the acetylation state of STAT3 at Lys-685. To probe the acetylation state of STAT3 Lys-685, we generated an acetyl-specific antibody against the Lys-685 site, designated anti-AcSTAT3 Lys685, and confirmed its specificity by immunoblotting of intact recombinant STAT3 proteins. Myc-tagged STAT3 WT, STAT3 YF or STAT3 KR mutant were expressed with or without HA-tagged p300 in 293T cells. The expressed STAT3 WT, STAT3 YF or STAT3 KR mutant protein was immunoblotted with anti-AcSTAT3 Lys685, anti-Myc or anti-HA antibodies. As shown in Fig. 1B, the STAT3 WT, STAT3 YF and STAT3 KR mutant proteins, with or without p300 coexpression, were expressed in equivalent amounts. The anti-AcSTAT3 Lys685 antibody failed to recognize the STAT3 KR mutant coexpressed with or without p300, indicating that the anti-AcSTAT3 Lys685 antibody specifically recognizes acetylated STAT3 at Lys-685. The similar results were obtained when we used anti-acetyl-lysine antibody (data not shown). Furthermore, overexpression of p300 showed an enhanced blot with anti-AcSTAT3 Lys685 antibody for both STAT3 WT and STAT3 YF, indicating that the acetylation state of STAT3 at Lys-685 is independent of the substitution of Tyr-705 to Phe. To further confirm the specificity of anti-AcSTAT3 Lys685 antibody, we tested the effect of overexpression of HDAC on the acetylation state of endogenous STAT3 at Lys-685. 293T cells were transfected with or without an increasing amount of HA-tagged HDAC3, and were treated with LIF. The transfected cells were lysed and immunoprecipitated with anti-STAT3 antibody, and Western blotting was performed with anti-AcSTAT3 Lys685 or anti-STAT3 antibody. As shown in Fig. 1C, the blot with anti-AcSTAT3 Lys685 showed a decreased acetylation state of STAT3, by overexpression of HDAC3. Therefore, these results show that anti-AcSTAT3 Lys685

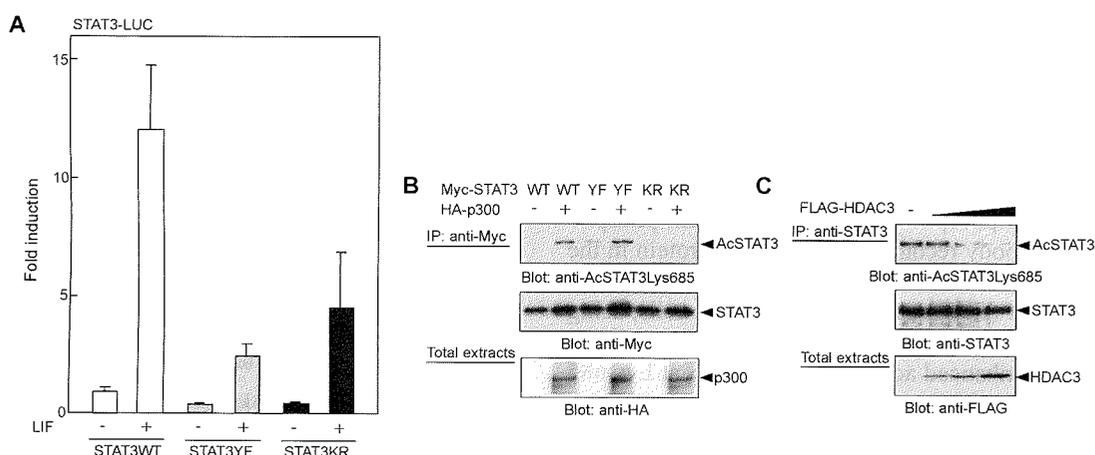


Fig. 1. Involvement of Lys-685 in STAT3 on STAT3 Activation and Detection of Its Acetylation State by an Acetyl-Specific Antibody

(A) 293T cells in a 24-well plate were transfected with STAT3-LUC (0.2 μ g) and/or indicated amounts (1 ng) of expression vector for STAT3 WT, STAT3 YF or STAT3 KR. Thirty-six hours after transfection, the cells were stimulated with LIF (50 ng/ml) for additional 8 h. The stimulated cells were harvested, and luciferase activities were measured. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D. (B) 293T cells (1×10^7) were transfected with or without Myc-tagged STAT3 WT, STAT3-YF or STAT3 KR mutants (10 μ g) and/or HA-tagged p300 (10 μ g). Forty-eight hours after transfection, the cells were lysed. Total cell lysates (1%) were blotted with anti-AcSTAT3 Lys685, anti-Myc or anti-HA antibody. (C) 293T cells (1×10^7) were transfected with or without the increasing amount of FLAG-HDAC3 (1, 3, 10 μ g). Forty-eight hours after transfection, the cells were treated with LIF (50 ng/ml) for 30 min. The cells were then lysed and immunoprecipitated with anti-STAT3 antibody and blotted with anti-AcSTAT3 Lys685 or anti-STAT3 antibody. Total cell lysates (1%) were blotted with anti-FLAG antibody.

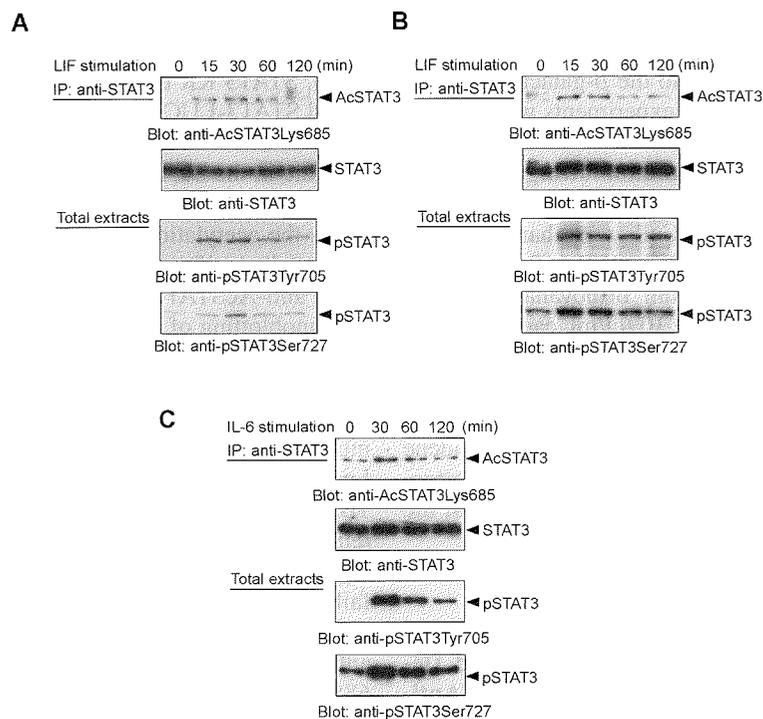


Fig. 2. LIF and IL-6 Induce Acetylation of Endogenous STAT3 at Lys-685

(A) 293T cells (1×10^7) were treated with LIF (50 ng/ml) for the indicated periods. The cells were then lysed and immunoprecipitated with anti-STAT3 antibody and blotted with anti-AcSTAT3 Lys685 or anti-STAT3 antibody. Total cell lysates (1%) were blotted with anti-pSTAT3 Tyr705 or anti-pSTAT3 Ser727 antibody. (B) HeLa cells (1×10^7) were treated with LIF (50 ng/ml) for the indicated periods. The cells were then lysed and immunoprecipitated with anti-STAT3 antibody and blotted with anti-AcSTAT3 Lys685 or anti-STAT3 antibody. Total cell lysates (1%) were blotted with anti-pSTAT3 Tyr705 or anti-pSTAT3 Ser727 antibody. (C) Hep3B cells (1×10^7) were treated with IL-6 (50 ng/ml) for the indicated periods. The cells were then lysed and immunoprecipitated with anti-STAT3 antibody and blotted with anti-AcSTAT3 Lys685 or anti-STAT3 antibody. Total cell lysates (1%) were blotted with anti-pSTAT3 Tyr705 or anti-pSTAT3 Ser727 antibody.

antibody can specifically recognize the acetylation state of STAT3 at Lys-685.

LIF and IL-6 Induce Acetylation of Endogenous STAT3 at Lys-685 We further assessed the acetylation state of Lys-685 in STAT3 after cytokine stimulation, using the anti-AcSTAT3 Lys685 antibody. We first tested LIF-induced acetylation of STAT3 at Lys-685 in 293T and HeLa cells. 293T and HeLa cells were treated with LIF for the indicated periods and then lysed. The cell lysate was immunoprecipitated with anti-STAT3 antibody, and Western blotting was performed with anti-AcSTAT3 Lys685, or anti-STAT3 antibody. Next, aliquots of the total cell lysate were also immunoblotted with anti-phospho STAT3 Tyr705 or anti-phospho STAT3 Ser727 antibody. As shown in Figs. 2A and B, the anti-AcSTAT3 Lys685 antibody recognized the STAT3 protein in the LIF-treated 293T and HeLa cells. In a similar time course, the anti-phospho STAT3 Tyr705 or anti-phospho STAT3 Ser727 antibody recognized the STAT3 protein in the LIF-treated 293T and HeLa cells. We also tested the IL-6-induced acetylation of STAT3 at Lys-685 in Hep3B cells, using the anti-AcSTAT3 Lys685 antibody. As shown in Fig. 2C, IL-6 stimulation also enhanced acetylation of STAT3 at Lys-685 in Hep3B cells. Therefore, we could detect LIF- or IL-6-induced acetylation of STAT3 at Lys-685 in 293T, HeLa and Hep3B cells, using the anti-AcSTAT3 Lys685 antibody. Furthermore, the anti-AcSTAT3 Lys685 antibody recognized acetylated STAT3 proteins in a similar time course with anti-phospho STAT3 Tyr705 or anti-phospho STAT3 Ser727 antibody.

Involvement of the PI3K/Akt Pathway in the Acetylation of STAT3 at Lys-685 Cytokine signaling utilizes

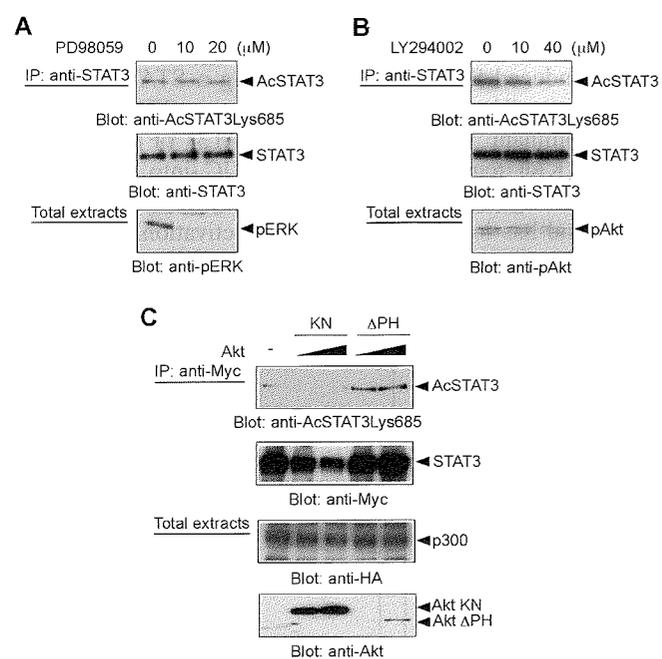


Fig. 3. Involvement of PI3K/Akt Pathway in the Acetylation of STAT3 at Lys-685

(A, B) Hep3B cells (1×10^7) were pretreated with DMSO or the indicated amount of PD98059 (A) or LY294002 (B) for 30 min. The pretreated cells were stimulated with IL-6 (50 ng/ml) for 30 min and then lysed and immunoprecipitated with anti-STAT3 antibody and blotted with anti-AcSTAT3 Lys685 or anti-STAT3 antibody. Total cell lysates (1%) were blotted with anti-pERK (A) or anti-pAkt antibody (B). (C) 293T cells (1×10^7) were transfected with Myc-tagged STAT3 and HA tagged p300 together with or without the increasing amount of Akt KN or Akt Δ PH (3, 10 μ g). Forty-eight hours after transfection, the cells were lysed and immunoprecipitated with anti-Myc antibody and blotted with anti-AcSTAT3 Lys685 or anti-Myc antibody. Total cell lysates (1%) were blotted with anti-HA or anti-Akt antibody.

not only the Jak/STAT pathway, but also the Ras/ERK or PI3K/Akt pathway. As shown in Fig. 1B, dominant negative STAT3 YF was also acetylated, and this suggests that acetylation of STAT3 at Lys-685 occurs independently with Jak/STAT signaling. We then tested the involvement of Ras/ERK and PI3K/Akt signaling on acetylation of STAT3 at Lys-685. To this end, we used pharmacologic inhibitors of Ras/ERK and PI3K/Akt signaling. We tested Ras/ERK inhibitor PD98059 and PI3K/Akt inhibitor LY294002. As shown in Fig. 3A, treatment with PD98059 had no effect on IL-6-induced acetylation of STAT3 at Lys-685 in Hep3B cells. However, treatment with LY294002 resulted in decreased acetylation of STAT3 at Lys-685 in Hep3B cells. These results suggest the involvement of PI3K/Akt, but not Ras/ERK signaling, in IL-6-induced acetylation of STAT3 at Lys-685. To confirm this, we tested the effect of dominant negative Akt KN with a substitution of the ATP-binding site (Lys-179 to Ala) and constitutively active Akt Δ PH, which is a membrane-targeted form of AKT lacking the PH domain,¹¹⁾ on the acetylation of STAT3 at Lys-685 by p300. Myc-tagged STAT3 WT and HA-tagged p300 were expressed with or without Akt KN or Akt Δ PH in 293T cells. The expressed STAT3 WT proteins were immunoprecipitated with anti-Myc antibody and blotted with anti-AcSTAT3 Lys685, anti-Myc or anti-HA antibodies. As shown in Fig. 3C, anti-AcSTAT3 Lys685 antibody failed to recognize STAT3 proteins when coexpressed with Akt KN. However, the anti-AcSTAT3 Lys685 antibody recognized STAT3 proteins strongly when coexpressed with Akt Δ PH, indicating that Akt activation stimulates acetylation of STAT3 at Lys-685, *via* p300. Therefore, PI3K/Akt signaling involves the acetylation of STAT3 at Lys-685 *in vivo*.

Concluding Remarks The results of the present study provide evidence that Lys-685 in STAT3 is an acetylation site for LIF and IL-6 stimulation, and it is involved in LIF-induced STAT3 activation. We further clarified that LIF or IL-6 induces acetylation of endogenous STAT3 at Lys-685 in 293T, HeLa and Hep3B cells, using an anti-AcSTAT3 Lys685 antibody. Moreover, we demonstrated the involvement of PI3K/Akt signaling in IL-6-induced acetylation of STAT3 at Lys-685.

Acetylation is known to be a dynamic post-translational modification of lysine residues. Proteins with intrinsic histone acetyltransferase activity are known to act as transcriptional co-activators by acetylating histones, thereby inducing an open chromatin conformation, which allows the transcriptional machinery access to promoters.¹²⁾ p300/CREB-binding protein (CBP) and P/CAF protein interact with a large number of transcription factors, as well as components of the basic transcriptional machinery. The androgen receptor, p53, GATA-1 and MyoD have been described as substrates of p300/CBP and P/CAF acetyltransferase activity, the acetylation of which affects DNA-binding affinity.^{13–16)} Acetylation also affects protein-protein interactions. For example, the NF- κ B RelA is acetylated by p300 on its entry into the nucleus, preventing its association with I κ B.¹⁷⁾ Acetylation of Smad7 stabilizes Smad7 protein and protects it from ubiquitin-mediated degradation through the proteasome pathway.¹⁸⁾

Cytokine-induced signaling, which activates acetyltransferase activity through p300/CBP, remains unclear. Recent studies have shown that insulin-like growth factor-I stimu-

lates acetylation of H3 and H4 histones in neurons, and that these effects are associated with activation of the PI3K/Akt pathway.¹⁹⁾ It has also been demonstrated that NF- κ B is activated by histone deacetylase inhibitors (HDIs) in a PI3K/Akt-dependent manner.²⁰⁾ HDI suberoylanilide hydroxamic acid (SAHA) stimulates NF- κ B transcription through a signaling pathway that involves activation of both Akt and p300.²¹⁾ SAHA stimulates Akt activity, which is required to phosphorylate p300 at Ser-1834.²²⁾ Furthermore, Akt-mediated phosphorylation of p300 dramatically increases its acetyltransferase activity, and is followed by increased acetylation of RelA/p65 at Lys-310, a modification that is required for full NF- κ B transcription. Therefore, monitoring the acetylation state of signaling molecules by stimuli is very important for investigating the activation state, as well as monitoring the phosphorylation state.

Recently, it has been also shown that STAT3 is acetylated at its N-terminal residue Lys-49 and Lys-87.²³⁾ STAT3 K49R/K87R mutant affected IL-6-induced transcriptional activity, but not IL-6-induced DNA binding activity, although STAT3 K685R affected both activities. These results suggest that the N-terminal acetylation of STAT3 is functionally different from that of STAT3 at Lys-685. Therefore, the detection of site-specific acetylation is very important to monitor the STAT3 activation state in various cellular conditions.

We believe that we have shown for the first time the involvement of PI3K/Akt signaling in the acetylation of STAT3 at Lys-685. Although further research is needed to elucidate in more detail the mechanisms involved in cytokine-induced acetylation of STAT3 through PI3K/Akt, the anti-AcSTAT3 Lys685 antibody produced in this study represents a powerful tool for clarifying the physiological roles of the acetylation state of STAT3 in normal and diseased conditions.

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