Journal of Biochemistry and Molecular Biology, Vol. 34, No. 6, November 2001, pp. 578-583

JOURNAL OF
Biochemistry and Molecular Biology
© BSRK & Springer-Verlag 2001

Cross-talk between STAT6 and Ras/MAPK Pathway for the IL-4-mediated T Cell Survival

Eui-Young So, Ji-Young Jang and Choong-Eun Lee*
Department of Biological Science and Institute for Basic Science, SungKyunKwan University, Suwon 440-746, Korea

Received 21 September 2001, Accepted 16 October 2001

As a prototypic Th1 *vs* Th2 cytokine, IFN-γ and IL-4 activate distinct STAT proteins, STAT1 and STAT6, respectively. In cytokine-producing Jurkat T cells, IL-4 is effectively rescued from cell death that is induced by dexamethasone, but IFN-γ failed to do so. Since the Ras/MAPK pathway is known to play an important role in cytokine-induced cell survival, we investigated the mechanism of T cell survival through the analysis of functional cross-talk between Ras/MAPK and distinct STAT proteins that are activated by IL-4 and IFN-γ. Although IL-4 and IFN-γ each induced the activation of STAT6 and STAT1 in Jurkat T cells, respectively, only IL-4 was capable of inducing MAPK. Along with tyrosine kinase inhibitors, MEK/MAPK inhibitors also caused a significant suppression of the IL-4-induced STAT6 activity. This suggests a positive regulation of STAT6 by MAPK during IL-4 signal transduction. Furthermore, transfection studies with dominant active (da) *vs* dominant negative (dn) Ras revealed that daRas, but not dnRas, selectively up-regulated the expression and activity of STAT6 with a concomitant increase in MAPK activity. These results, therefore, suggest that there is a functional cross-talk between the Ras/MAPK and Jak/STAT6 pathways, which may have a role in the IL-4-induced T cell survival.

Keywords: IL-4, Ras/MAPK, Signal cross-talk, STAT6, T cell survival

Introduction

With the recent progress in the elucidation of the cytokine signal transduction, Jak/STAT and Ras/MAPK have been established as two major intracellular signaling pathways that control cytokine-induced cell survival, proliferation, and differentiation (Max, 1993). In fact, various cytokines, growth factors, and hormones are now found to activate both the Jak/STAT and Ras/MAPK pathways, which affect diverse facets of cell growth signaling processes (Boulton *et al.*, 1995; Yamanaka *et al.*, 1996).

Unlike many other cytokines that regulate immune cell growth, relatively little is known about IL-4 signal transduction that leads to cell growth response. It was thought that the signaling pathways involved in the IL-4-induced cell survival/proliferation *vs* differentiation are rather distinct. Upon IL-4 receptor activation by ligand binding, both the insulin receptor substrate (IRS)-1/2 and STAT6 bind to a specific motif on the IL-4 receptor, called the insulin-interleukin-4 receptor (I4R) motif (Wang *et al.*, 1998). Subsequently, the activation of the IRS-1/2-mediatied PI3K and MAPK activation is implicated in IL-4-induced cell survival, whereas the Jak1-dependent STAT6 activation is thought to be responsible for the induction of gene expression and differentiation (Mikita *et al.* 1996; Nelms *et al.*, 1999). In contrast, the IFN-γ-induced STAT1 activation is important, not only in the regulation of the IFN-γ-induced gene expression and differentiation, but also in the induction of growth arrest and apoptosis of target cells (Xu *et al.*, 1998; Suk *et al.*, 2001).

In the Jurkat T cells that are capable of producing both Th1 and Th2 cytokines, IL-4 and IFN-γ display opposing effects. IL-4 mediates cell survival from the dexamethasone-induced apoptosis, whereas IFN-γ promotes cell death. Since Ras/MAPK pathways are known to play an important role in cytokine-induced cell survival, we are interested in whether there is a selective cross-talk between Ras/MAPK and distinct STAT proteins that are activated by IFN-γ and IL-4, which would ultimately produce differential growth-regulating effects of the Th1 *vs* Th2 cytokines in these cells.

Here we report that IL-4, but not IFN-γ, selectively induces MAPK activity in Jurkat T cells. There is also a functional cross-talk between the Jak/STAT6 and Ras/MAPK pathways. Through transfection studies we have also demonstrated that Ras/MAPK components positively regulate the expression and activity of STAT6. The induction of MAPK activation by

*To whom correspondence should be addressed.
Tel: 82-31-290-7006; Fax: 82-31-290-7015
E-mail: celee@yurim.skku.ac.kr

IL-4 and the selective cross-talk between the Jak/STAT6 and Ras/MAPK pathway would contribute to IL-4-mediated cell survival and subsequent differentiation to Th2 cells that are induced upon the IL-4 treatment.

Materials and Methods

Cell culture Jurkat T cells were maintained in a RPMI media supplemented with 10% FBS (Life Technologies Inc., Grand Island, USA), 10 mM HEPES, 2 mM L-glutamine, 5×10^{-5}M β-ME, 50 μg/ml gentamicin, and 50 μg/ml amphotericin B (Sigma, St Louis, USA). The 293 T cells were maintained in DMEM supplemented with 5% FBS. Recombinant human IL-4 (R+D systems, Minneapolis, USA), IFN-γ (R & D systems), or dexamethasone (Sigma) was added to the cells as indicated. The cells were cultured in humidified 5% CO_2 at 37°C.

MAPK assays Cells were stimulated with cytokines for 5 to 60 min, and the total lysates were prepared as described (So *et al.*, 2000). The lysates were fractionated on a 10% SDS-PAGE under denaturing conditions. Then the gel was transferred to PVDF membranes. The membrane was immunoblotted with a polyclonal anti-phospho-Erk antibody (Upstate Biotechnologies, Inc), then subsequently with an anti-Erk antibody. The blots were developed using an ECL system (Amersham). An *in vitro* MAPK assay was also performed with the cytokine-treated lysates. The total lysates were immunoprecipitated (Park *et al.*, 2000) with an anti-MAPK (Erk1/2) antibody and anti-rabbit IgG agarose. The precipitates were then incubated with myelin basic protein (MBP) as a substrate. Then the phosphorylation of MBP was analyzed on a 10% SDS-PAGE by autoradiography.

STAT activation assays The cells were treated with cytokines for 30 min. The nuclear extracts were prepared to perform EMSA by using the FcγRI GAS oligomer or FcεRII GAS oligomer as a probe for STAT1 or STAT6, respectively (Koh *et al.*, 2000; Song *et al.*, 2001). Where indicated, the Jurkat T cells were pre-treated with protein kinase inhibitors for 30 min prior to the cytokine stimulation. The binding of activated STATs to the labeled GAS probe was then analyzed by the mobility shift from the free probe on a 5% PAGE in a 0.5X TBE buffer. In addition, in order to assess the activation status of the STAT protein, the tyrosine phosphorylation level of STAT6 was analyzed by immunoblotting using polyclonal anti-phosphotyrosine-STAT6 antibodies (UBI) as described (So *et al.*, 2000).

Apoptosis study Phytohemagluttin (PHA, Sigma)-stimulated or unstimulated Jurkat T cells were treated with IL-4 or IFN-γ in the presence or absence of dexamethasone. The dexamethasone-induced apoptosis was then monitored by performing a DNA fragmentation analysis on a 2% agarose gel.

Transfection and Western blot The 293 T cells were plated at a density of 2×10^5 cells/well in 6-well plates. The cells were transfected with a cDNA construct of STAT6, daRas, or dnRas by Fugene 6 reagent (Böheringer Mannheim) according to the manufacturer's instructions. Two days (48 h) after transfection, the cells were harvested. Then the total lysates were prepared. The expression levels of da/dn Ras and STAT6 were analyzed by immunoblotting using respective antibodies (Kim *et al.*, 2000).

Results and Discussion

Differential effects of IL-4 and IFN-γ on the Jurkat cell apoptosis and survival As a transformed T helper cell line, Jurkat cells produce, in addition to IL-2, both Th1 and Th2 cytokines upon appropriate stimulation. PHA stimulation of Jurkat cells leads to cellular activation and IL-2 production, which promotes cell growth. The cells, however, can be induced to undergo apoptosis upon treatment with a synthetic steroid, dexamethasone, as shown by the significant fragmentation of nuclear DNA (Fig. 1. Panel A, lanes 1 and 4). IL-4 effectively rescued these cells from the dexamethasone-induced apoptosis, but IFN-γ failed to do so. In fact, IFN-γ alone was capable of inducing cell death, even in the absence of dexamethasone (Fig. 1 Panel A, lanes 3). Such differential effects of IL-4 and IFN-γ on the apoptosis of T cells were also observed even when unstimulated Jurkat cells were used; IL-4 rescued the cells from the apoptosis while IFN-γ did not (Fig 1, Panel B). The results indicate that IL-4 and IFN-γ differentially regulate the signaling pathway that leads to the growth/survival response in T cells.

Differential activation of STATs and MAPK by IL-4 and IFN-γ In order to delineate the underlying mechanism of the

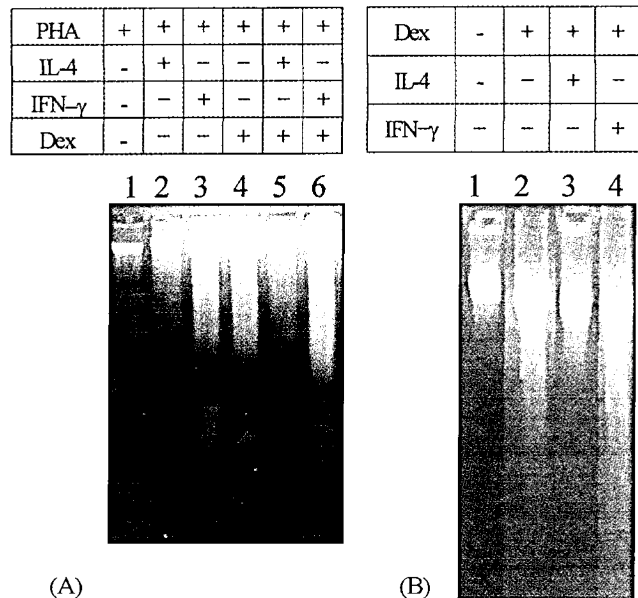

Fig. 1. Differential effects of IL-4 and IFN-γ on the dexamethasone-induced apoptosis of Jurkat T cells. Jurkat T cells (2×10^7) were treated with PHA (5 μg/ml) for 4 h (Panel A) or left untreated (Panel B). The cells were washed, then IL-4 (10 ng/ml), IFN-γ (10 ng/ml), and dexamethasone (Dex, 10 μM) were added and cultured for 24 h. Nuclear DNAs were extracted and analyzed on a 2% agarose gel.

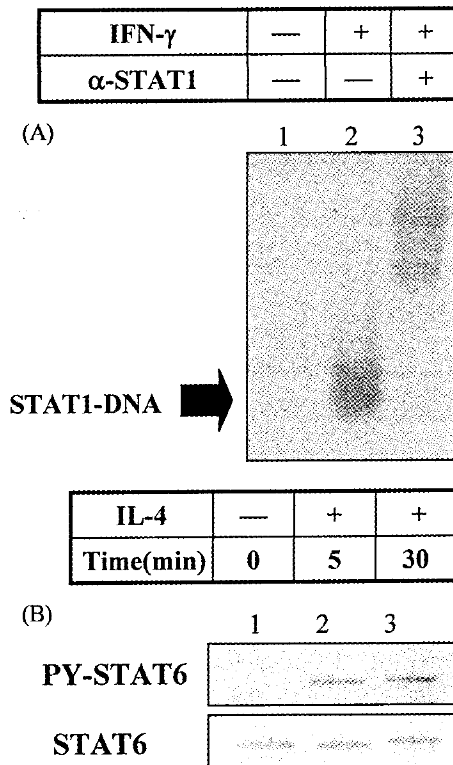

Fig. 2. IFN-γ and IL-4 induce STAT1 and STAT6 activity, respectively in Jurkat T cells. Panel A: Jurkat T cells were treated with media alone or with IFN-γ (10 ng/ml) for 30 min. Nuclear extracts were then prepared and subjected to EMSA using the FcγRI GAS probe as described in the text. Anti-STAT1 antibodies were incubated with the extracts for 30 min prior to the binding reaction with the labeled probe. Panel B: Jurkat T cells were treated with media alone or with IL-4 (10 ng/ml) for the indicated durations. Total cell lysates were prepared and subjected to immunoblotting with anti-phosphotyrosine STAT6 antibodies (Top). The blot was then stripped and reprobed with anti-STAT6 antibodies (Bottom).

differential action of IL-4- and IFN-γ on T cell survival, we analyzed the IL-4- or IFN-γ-induced STAT activation profile in these cells as a primary signaling molecule that regulates the T cell growth response.

As is known for other cell systems, the treatment of Jurkat T cells with IFN-γ induced STAT1 activation (Park *et al.*, 1998; Schindler *et al.*, 1992). The nuclear extracts of IFN-γ-treated T cells contained the activity binding to FcγRI GAS, the STAT1-recognition sequence (Fig. 2 Panel A, lanes 1 and 2). The identity of the complex was confirmed to be STAT1 by a supershift assay using anti-STAT1 antibodies (Fig. 2 Panel A, lane 3). Also, IL-4 induced STAT6 activation in these cells. This is shown by a noticeable increase in tyrosine phosphorylation of STAT6 (Quelle *et al.*, 1995) in a time-dependent manner, as demonstrated by immunoblotting with anti-phosphotyrosine STAT6 antibodies (Fig. 2, Panel B). These results demonstrate that IL-4 and IFN-γ both activate the Jak/STAT pathway in Jurkat cells, which may regulate the growth response via distinct mechanisms.

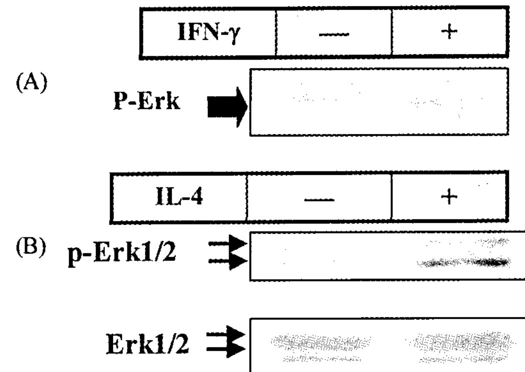

Fig. 3. IL-4 but not IFN-γ induces MAPK activation in Jurkat T cells. Panel A: Jurkat cells were treated with media alone or IFN-γ (10 ng/ml) for 20 min. The lysates were prepared and subjected to immunoblotting with anti-phosphothreonine-Erk antibodies. Panel B: Jurkat cells were treated with media alone or IL-4 (10 ng/ml) for 20 min. The lysates were subjected to immunoblotting with anti-phosphothreonine Erk antibodies (Top). The stripped membrane was subsequently used for blotting with anti-Erk antibodies (Bottom).

While STAT1 is implicated in cytokine-induced growth arrest, the role of STAT6 in the growth-promoting process has not been defined (Zamorano *et al.*, 1998). Thus, next we examined whether IL-4 or IFN-γ induces MAPK, which is thought to be involved in cytokine-induced cell survival mechanisms (Wang *et al.*, 1993). As in Fig 3, the treatment of the Jurkat T cells with IL-4 at doses effective for the IL-4-mediated T cell survival resulted in a significant increase in phospho-threonine Erk 1/2 levels at 30 min. This represents a strong induction of MAPK activity by IL-4. Among the two known isoforms of Erk, IL-4 preferentially activated p42 Erk2 more than p44 Erk1 (Fig 3, Panel B). In contrast, IFN-γ did not induce Erk MAPK activation by 30 min (Fig, Panel A). The prolonged incubation of these cells with IFN-γ up to 1 h at doses used for the induction of apoptosis, was still ineffective for MAPK activation (data not shown). The treatment of Jurkat T cells with an Erk inhibitor, PD98059 (Alessi *et al.*, 1995), blocked the effect of IL-4 on the rescue of these cells from the dexamethasone-induced apoptosis (data not shown). The results suggest a role of MAPK in the IL-4-induced Jurkat T cell survival.

Positive regulation of STAT6 by Ras/MAPK Components
Since IL-4, but not IFN-γ, induced cell survival and the concomitant activation of STATs and Erk MAPK in Jurkat T cells, we were interested to examine whether there is a selective cross-talk between MAPK and STAT activation pathway, which may influence the IL-4-induced T cell rescue from the apoptosis. Thus, we initially examined whether the activity of STAT6 is regulated by MAPK-dependent pathways by using various protein kinase inhibitors.

As shown in Fig 4, the IL-4-induced STAT6 activity was

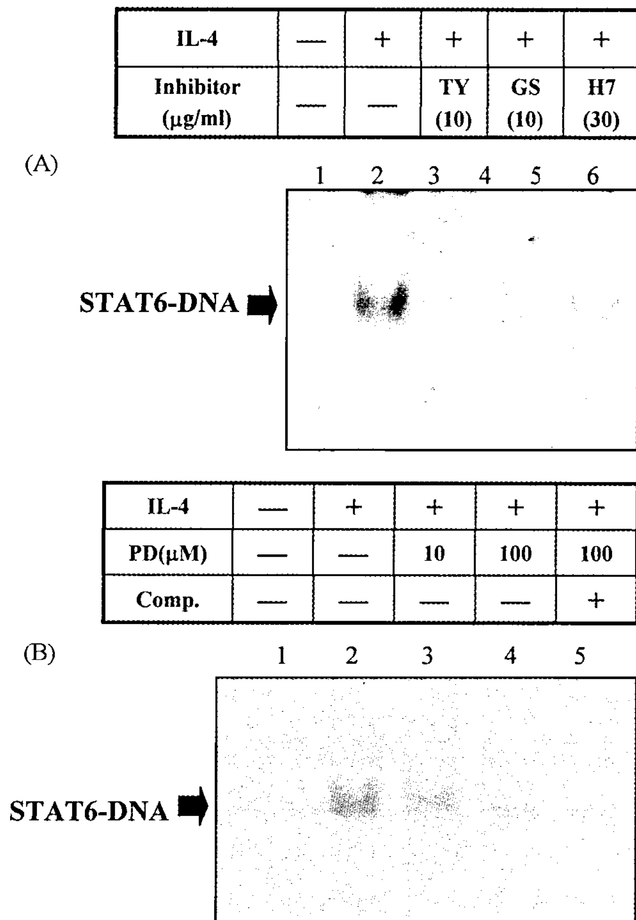

Fig. 4. Suppression of the IL-4-induced STAT6 activity by inhibitors of PTK and MEK/MAPK. EMSA was performed with T cells treated with IL-4 (5 ng/ml) for 30 min in the absence or presence of specific protein kinase inhibitors, tyrphostin (TY), genistein (GS), H-7, or PD98059 (PD) using the FcεRII GAS probe that contained a STAT6 binding site. Inhibitors or competitor oligomers (comp, 100×) were added 30 min prior to the IL-4 treatment.

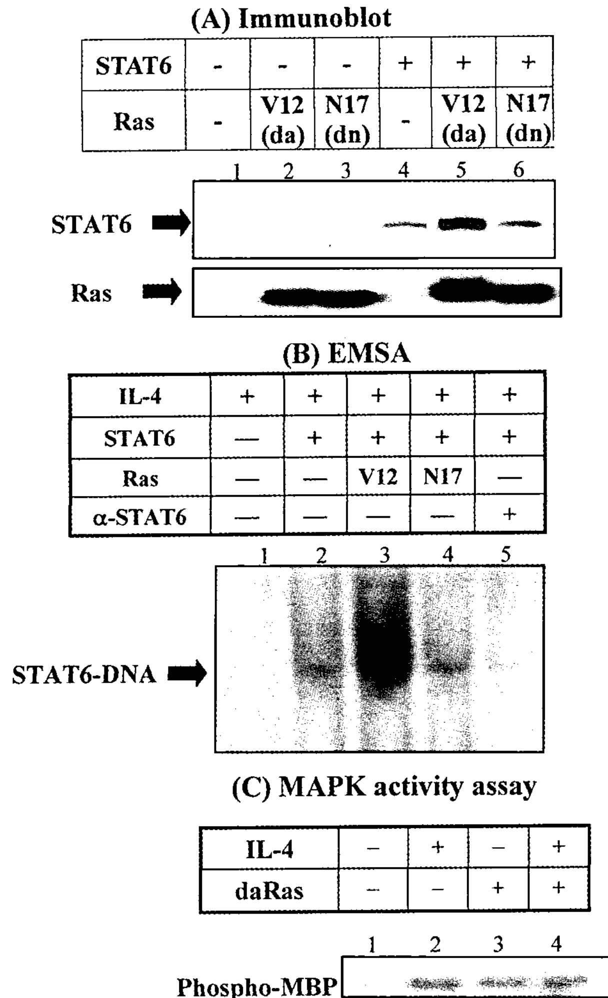

Fig. 5. Regulation of expression and activity of STAT6 by da/dn Ras co-transfection. The 293 T cells were transfected with vector alone (-), daRas, or dnRas with or without STAT6 co-transfection. The expression levels of STAT6 and Ras were analyzed by Western blot with respective antibodies (Panel A). The extracts of the transfected cells were also analyzed for STAT6 activation profile upon IL-4 (10 ng/ml) treatment for 30 min by performing EMSA as in Fig. 4 (Panel B). The *in vitro* MAPK assay was performed with the extracts of the transfected cells using MBP as a substrate with or without the IL-4 (10 ng/ml) treatment for 15 min.

completely abrogated by tyrosine kinase inhibitors, tyrphostin, or genistein (Akiyama *et al.*, 1987). H7, at concentrations effective for the specific inhibition of PKC (Hidaka *et al.*, 1984), did not effectively suppress the IL-4-induced STAT6 activity (Fig 4, Panel A). This demonstrates that the IL-4-induced STAT6 activation proceeds via tyrosine kinase (Jak)-dependent yet PKC-independent pathways, as indicated in earlier studies (Lee *et al.*, 1993; Quelle *et al.*, 1995). Noticeably an inhibitor of Erk, PD98059, significantly down-regulated the IL-4-induced STAT6 binding to the target DNA in a dose-dependent manner. This suggests that STAT6 activation is regulated, at least in part, by the Erk MAPK-dependent pathway in these T cells (Fig 4, Panel B).

To demonstrate more directly the regulation of STAT6 though the Ras/MAPK pathway, we utilized the 293 T cell system, which is deficient in the constitutive expression of STAT6 (Fig 5, Panel A, lanes 1-3). We observed that the co-transfection of daRas and STAT6 in these cells resulted in an elevated expression and up-regulation of STAT6 activity, as compared to STAT6 transfection alone. No such positive effects on the STAT6 activation and expression were obtained with the dnRas co-transfection (Fig 5, Panels A and B). It should be noted that in the daRas-transfected cells, the magnitude of the elevation in STAT6 activity is far greater than the increase in the expression level of transfected STAT6. This indicates that daRas not only caused the increase in the STAT6 protein level, but also induced the elevation of the specific activity of STAT6 for its binding to the target DNA.

The MAPK assay data with the corresponding lysates obtained from daRas and/or STAT6-transfected cells clearly demonstrated that daRas transfection indeed resulted in the up-regulation of MAPK activity (Fig 5, Panel C). Transfection studies performed with Jurkat T cells produced basically the same results (data not shown).

These provide evidence that the daRas-induced elevation of STAT6 activity is likely due to the Ras-dependent activation of MAPK and that Ras/MAPK module components also positively regulate STAT6 activity. In these cells, however, the co-transfected STAT1 was not differentially modulated by da/dn Ras (data not shown). Considering the previous finding that IFN-γ can induce apoptosis of target cells by the up-regulation of the Fas expression *via* STAT1-dependent mechanisms (Xu *et al.*, 1998), the inability of MAPK induction by IFN-γ in those cells that are coupled with the activation of STAT1 may play a part in the IFN-γ-induced cell death that was observed in this study.

In conclusion, the data from the present work suggest that there is a positive regulation of STAT6 by Ras/MAPK components, as was implicated in recent studies (Horvai *et al.*, 1999; Yamashita *et al.*, 1999). The functional cross-talk between STAT6 and MAPK during IL-4 signal transduction is likely involved in the IL-4-induced T cell survival from the dexamethasone-induced apoptosis. Thus, in addition to the well-established role for the IL-4-induced Th2 differentiation (Nakanishi *et al.*, 1996), STAT6 may participate in the IL-4-mediated T cell survival and proliferation.

Acknowledgments We thank Dr. Groner at Göthe University for the kind provision of the STAT6 cDNA construct. This study was supported in part by research grants from KOSEF (#R01-1999-00-138), MOST (Nuclear Basic Research Project), and KFDA. E. Y. So and J. Y. Jang were supported by the Brain Korea 21 Program.

References

Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuga, M. and Fukami Y. (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinase. *J. Biol. Chem.* **262**, 5592-5595.

Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995) PD98059 is a specific inhibitor of the inhibitor of the activation of mitogen-activated protein kinase *in vitro* and *in vivo*. *J. Biol. Chem.* **270**, 27489-27494.

Boulton, T. G., Zhong, Z., Wen, Z., Darnell, J. E. Jr., Stahl, N. and Yancopoulos, G. D. (1995) STAT3 activation by cytokines utilizing gp130 and related transducers involves a secondary modification requiring an H7-sensitive kinases. *Proc. Natl. Acad. Sci. USA* **92**, 6915-6919.

Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. (1984) Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide-dependent protein kinase and protein kinase C. *Biochemistry* **23**, 5036-5041.

Horvai, A. E., Xu, L., Korzus, E., Brand, G., Kalafus, D., Mullen, T. M., Pose, D. W., Resenfeld, M. G. and Glass, G. K. (1997) Nuclear integration of Jak/STAT and Ras/AP-1 signaling by CBP and p300. *Proc. Natl. Acad. Sci. USA* **94**, 1074-1079

Kim, S-W., Pyo, S-N. and Rhee, D-K. (2000) Characterization of dnaK mutants in *Streptococcus pneumoniae*. *J. Biochem. Mol. Biol.* **33**, 75-81.

Koh, H. J., Park, H. H. and Lee, C. E. (2000) Regulation of IgE and type II IgE receptor expression by insulin-like growth factor-1: Role of STAT6 and NF-κB. *J. Biochem. Mol. Biol.* **33**, 454-462.

Lee, C. E., Yoon, S. R. and Pyun, K. H. (1993) Interleukin-4 signals regulating CD23 gene expression in human B cells: Protein Kinase C-independent signaling pathways. *Cell. Immunol.* **146**, 171-185.

Leonard, W. J. and O'Shea, J. J. (1998) Jaks and STATs: biological implications. *Annu. Rev. Immunol.* **16**, 293-322.

Max, J. (1993) Two major signal pathways linked. *Science* **262**, 988-990.

Mikita, T, Campbell, D., Wu, P. G., Williamson, K. and Schindler, C. (1996) Requirement for IL-4-induced gene expression and functional characterization of STAT6. *Mol. Cell. Biol.* **16**, 5811-5820.

Nakanishi, K., Yoshida, N., Kishimoto, T. and Akira, S. (1996) Essential role of STAT6 in IL-4 signaling . *Nature* **380**, 627-630.

Nelms, K., Keegan, A. D., Zamorano, J. Ryan, J. J. and Paul, W. E. (1999) The IL-4 receptor: signaling mechanisms and biologic functions. *Annu. Rev. Immunol.* **17**, 701-738.

Park, H. J., So, E. Y. and Lee, C. E. (1998) Interferon-γ-induced factor binding to the IL-4RE of CD23 promoter. *Mol. Immunol.* **35**, 239-247.

Park, S., Nelson, T. J., Alkon, D. L. and Kim, J. (2000) Functional characterization of the squid calexcitin-2, a calcium and GTP-binding protein. *J. Biochem. Mol. Biol.* **33**, 391-395.

Quelle, F. W., Shimoda, K., Thiefelder, W., Fisher, C., Kim, A., Ruber, S. M., Cleveland, J. L., Poerce, J. H., Keegan, A. D., Nelms, K., Paul, W. E. and Ihle, J. N. (1995) Cloning of murrine STAT6 and human STAT6, STAT protein that are tyrosine phosphorylated in response to IL-4 and IL-13, but are not required for mitogenesis. *Mol. Cell. Biol.* **15**, 3336-3343.

Schindler, C., Shcii, K., Preziose, V. R. and Darnell, J. E. Jr. (1992) Interferon-dependent tyrosine phosphorylation of a cytoplasmic transcription factor. *Science* **257**, 809-813.

So, E. Y., Park, H. H. and Lee, C. E. (2000) Interferon-γ and interferon-a post-transcriptionally down-regulate the interleukin-4-induced interleukin-4 receptor gene expression. *J. Immunol.* **165**, 5472-5479.

Song, J. H., Park, H. H., Park, H. J., Han, M. Y., Kim, S. H. and Lee, C. E. (2001) Role of STAT3 as a molecular adaptor in cell growth signaling: Interaction with Ras and other STAT proteins. *J. Biochem. Mol. Biol.* **34**, 484-488.

Suk, K., Kim, S., Kim, Y. H., Kim, K. A., Chang, I., Yagita, H., Shong, M. and Lee, M. S. (2001) IFN-γ/TNF-α synergism as the final effector in autoimmune diabetes: a key role for STAT1/IFN regulatory factor-1 pathway in pancreatic beta cell death. *J. Immunol.* **166**, 4481-4489.

Wang, L. W., Myers, M. G., Sun, X. J., Aaronson, S. A., White, M. and Pierce, J. H. (1993) IRS-1: essential for insulin-and IL-4-stimulated mitogenesis in hematopoietic cells. *Science* **261**, 1591-1594.

Wang, H. Y., Zamorano, J. and Keegan, A. D. (1998) A role for the insulin-interleukin-4 receptor motif of the IL-4 receptor alpha chain in regulating activation of IRS-2 and STAT6 pathway: Analysis by mutagenesis. *J. Biol. Chem.* **273**, 9898-9905.

Xu, X., Fu, X. Y. Plate, J. and Chong, A. S. (1998) IFN-gamma induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and Fas L expression. *Cancer Res.* **58**, 2832-2837.

Yamanaka, Y., Nakajima, K., Fukada, T., Hibi, M. and Hirano, T. (1996) Differentiation and growth arrest signals are generated through the cytoplasmic region of gp130 that is essential for Stat3 activation. *EMBO J.* **15**, 1557-1565.

Yamashita, M., Kimura, M., Schimizu, C., Tada, T., Perlmutter, R. M. and Nakayama, T. (1999) T cell antigen receptor-mediated activation of the Ras/MAPK pathway controls IL-4 receptor function and type 2 helper T cell differentiation. *Proc. Natl. Acad. Sci. USA* **96**, 1024-1029.

Zamorano, J. and Keegan, A. D. (1998) Regulation of apoptosis by tyrosine-containing domain of IL-4R alpha: Y497 and Y713, but not the STAT6-docking tyrosine; signal protection from apoptosis. *J. Immunol.* **161**, 850-858.