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Short communication

Role of STAT3 as a Molecular Adaptor in Cell Growth Signaling: Interaction with Ras and other STAT Proteins

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STATs are proteins with a dual function: signal transducers in the cytoplasm and transcriptional activators in the nucleus. Among the six known major STATs (STAT1-6), STAT3 has been implicated in the widest range of signaling pathways that regulate cell growth and differentiation. As a part of our on-going investigation on the pleiotropic functions of STAT proteins, we examined the role of STAT3 as a molecular adaptor that links diverse cell growth signaling pathways. We observed that STAT3 can be specifically activated by multiple cytokines, such as IL-3, in transformed fibroblasts and IL-4 or IFN-γ in primary immune cells, respectively. The selective activation of STAT3 in H-ras-transformed NIH3T3 cells is associated with an increased expression of phosphoserine STAT3 in these cells, compared to the parental cells. Notably phosphoresine-STAT3 interacts with oncogenic ras, shown by immunoprecipitation and Western blots. The results suggest the role of STAT3 in ras-induced cellular transformation as a molecular adaptor linking the Jak/STAT and Ras/MAPK pathways. In primary immune cells, IL-4 and IFN-γ each induced (in addition to the characteristic STAT6 and STAT1 homodimers) the formation of STAT3-containing complexes that bind to GAS probes, which correspond to the Fcε RII and Fcγ RI promoter sequences, respectively. Since IL-4 and IFN-γ are known to counter-regulate the expression of these genes, the ability of STAT3 to form heterodimeric complexes with STAT6 or STAT1 implies its role in the fine-tuned control of genes that are regulated by IL-4 and IFN-γ.

Keywords: Cellular transformation, Cytokines, Differentiation, Growth signaling, Signal transducers and activators of transcription (STAT) 3

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Introduction

Cell growth signaling is mediated by growth factors and cytokines, which utilize intra-cellular signal transduction pathways that involve the activation of cytoplasmic signaling molecules and nuclear transcriptional factors. When these bind to specific DNA sequence motifs, they regulate the expression of diverse genes. Recently a family of signaling proteins with the dual function of signal transduction and transcriptional activation (namely STATs) were recognized. They play a key role in the cytokine/growth factor receptor-activated signaling processes (Darnell, 1997; Leonard and O'Shea, 1998).

Among the known STATs (STAT1-6), STAT3 has been implicated in the most diverse signaling pathways. It is activated by multiple factors, such as IL-2, IL-6, EGF, IFN-α, LIF, CNTF, and oncostatin M (Boulton *et al.*, 1995; Yamanaka *et al.*, 1996; Wishingrad *et al.*, 1997). While specific STAT knock-out mice have been generated that display specific defects that are associated with the suggested function of the deleted STAT gene, STAT3 knock-out animals have not been successfully generated. This implies the essential and pleiotropic role of STAT3 in embryonic development, as well as cell growth and differentiation (Takeda *et al.*, 1997).

Diverse roles of STAT3 in cell growth signaling were suggested in previous findings. First, the full activation of STAT3 requires, in addition to the tyrosine phosphorylation at Y701, the serine phosphorylation at S727. This indicates that STAT3 acts as a linker molecule, which connects both the tyrosine kinase and serine/threonine kinase-dependent signaling pathways (Wen *et al.*, 1995; Zhang *et al.*, 1995). Second, selective activation of STAT3 was reported in the virus- or oncogene-induced cellular transformation. In addition, the specific association of STAT3 with c-src or c-jun suggests the role of STAT3 in cellular transformation (Migone *et al.*, 1995; Chaturvedi *et al.*, 1998). Finally, the simultaneous molecular interaction of STAT3 with the cytokine receptor

(IFN-αR) and its down-stream effector (PI3K) was observed. This suggests the role of STAT3 as an adaptor in certain growth signaling pathways (Pfeffer *et al.*, 1997).

Based on these findings, we explored the role of STAT3 as a molecular linker, which connects distinct growth signaling pathways that regulate cellular proliferation, differentiation, and transformation.

Materials and Methods

Cell culture Human primary lymphocytes were obtained by purification from freshly excised tonsils using Ficoll-Hypaque (d = 1.077, Sigma, St. Louis, USA) by density gradient centrifugation, and cultured in a RPMI media that contained 10% FBS (Hyclone) in humidified 5% CO_2 at 37°C (Koh *et al.*, 2000). NIH3T3 fibroblasts, and its H-ras-transformed fibroblast cell lines, were maintained in DMEM. Interleukins (IL-3, IL-4, IL-6) and interferon-γ (R+D Systems, Minneapolis, USA) were treated to cells under serum-free conditions.

Electrophoretic mobility shift assays (EMSA) The nuclear cell extract preparation for EMSA was performed by Park *et al.* (1998). GAS oligomers (Fcε RII GAS: 5'GGGTGAATTTCTAAG AAAGGG3', and Fcγ RI GAS: 5'TGAGATGTATTTCCC-AGAA AAG3') were synthesized (Bioneer, Cheongwon, Korea) and labeled with [α-^{32}P] dCTP (3000 Ci/m mol, NEN) by Klenow. The nuclear extracts (5-10 μg) were incubated with the labeled oligomer in a binding buffer that contained 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM $NaVO_3$, 10 mM NaF, and 2 μg poly(dI) · (dC) for 20 min at room temperature. The mobility shift of the oligomer was then analyzed by 5% PAGE in a 0.5× TBE buffer.

Immunoprecipitation and Western blots Cytoplasmic extracts from the control or cytokine- treated cells were prepared and immuno-precipitated with anti-Ha-ras Ab (R+D systems) as described (Lee *et al.*, 2001). The precipitates were subjected to denaturing 10% SDS-PAGE, after which the gels were blotted onto a nitro-cellulose membrane. The membranes were probed with anti-STAT3 or anti-phosphoserine STAT3 antibodies (Upstate Biotechnology Inc.) and developed employing the enhanced chemiluminescence (ECL) system (Amersham).

Results and Discussion

Since the activation of STAT3 was reported in certain virus- or oncogene-induced cellular transformations, such as c-src (Migone *et al.*, 1995; Chaturvedi *et al.*, 1998), we first examined the role of STAT3 activation in ras-transformed cells. In v-H-*ras*(V12)-transformed fibroblasts, we observed a selective activation of STAT3 upon stimulation with IL-3. The nuclear extracts of the ras-transformed cells, but not that of the parental cells, contained the consensus GAS (Fcγ RI GAS: STAT1/3 recognition sequence)-binding activity (Fig. 1 Panels A vs. B, band I). The supershift pattern indicated that this

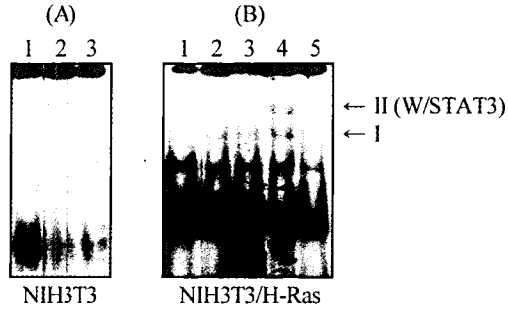

Fig. 1. Selective activation of STAT3 in ras-transformed fibroblasts. Panel A: NIH3T3 cells (1×10^7) were treated with media alone (lane 1), 3 ng/ml IL-3 (lane 2), or 30 ng/ml IL-3 (lane 3), for 15 min. Nuclear extracts were prepared and analyzed by EMSA using Fcγ RI GAS (consensus STAT binding sequence) as a probe. Panel B: Ras-transformed NIH3T3 cells (1×10^7) were treated with media alone (lane 1), 30 ng/ml IL-3 (lanes 2 to 5) for 15 min. Nuclear extracts were preincubated with anti-STAT1 Ab (lane 3), anti-STAT3 Ab (lane 4), or control IgG (lane 5) prior to the addition of the labeled Fcγ RI probe.

complex contained a protein that specifically reacted with anti-STAT3, but not with anti-STAT1 antibodies (Fig. 1 Panel B, lanes 3 vs. 4, band II). The activation of STAT3 in these transformed cells was associated with an increased level of 89 kDa protein and the distinct appearance of the 91 kDa protein, both of which reacted with the polyclonal anti-STAT3 antibodies (Fig. 2-A).

Such an elevated STAT3 expression seemed, at least in part, due to the increased STAT3 mRNA levels that were found in the ras-transformed cells, as analyzed by Northern blot (data not shown). Interestingly, anti-H-ras Ab specifically co-immunoprecipitated the 91 kDa, but not the 89 kDa STAT3 (Fig. 2-B). Furthermore, the 91 kDa STAT3 reacted with the anti-phosphoserine STAT3 antibody (Fig. 2-C). These results suggest that there is a specific molecular interaction between the 91 kDa phosphoserine STAT3 and Ras. Also, STAT3 may be a target of the Ras/MAPK module enzyme in these transformed cells, as previously implicated in other cell systems (David *et al.*, 1995; Ihara *et al.*, 1997). It should be noted that the increased expression, interaction with Ras, and serine phosphorylation of STAT3 are not generally dependent on the cytokine (IL-3) stimulation. Rather, they appear as an inherent property of the ras-transformed cells. The constitutive serine phosphorylation of STAT3 may render STAT3 more responsive to full activation upon cytokine stimulation, which induces tyrosine phosphorylation by the action of Jak kinases. In fact, both the serine and tyrosine phosphorylation are required for the maximal activation for STAT3 (Wen *et al.*, 1995; Zhang *et al.*, 1995). Since STAT3 has been widely implicated in regulating cell growth signaling in diverse transformed cells, one of the mechanisms of proliferation in these ras-transformed cells may include the up-regulation of the protein expression and serine phosphorylation of STAT3 by Ras/MAPK-dependent pathways.

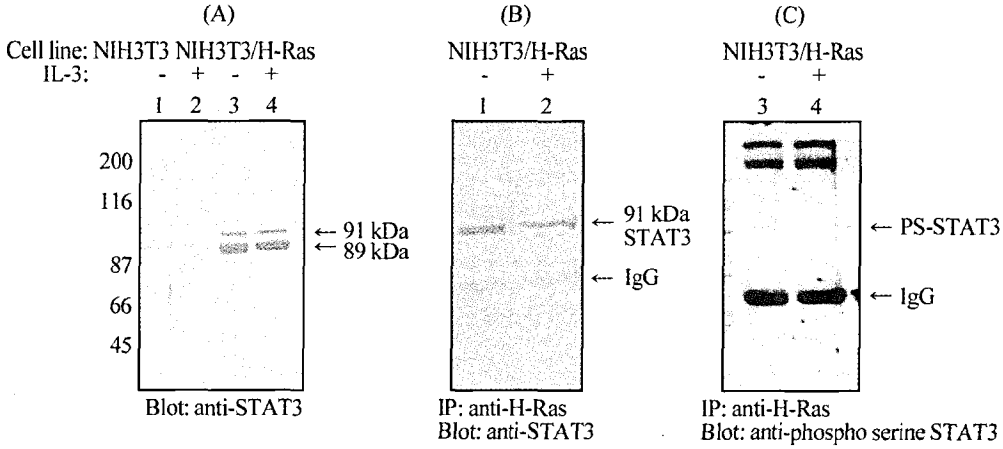

Fig. 2. Increased expression of STAT3 and selective association of phosphoserine-STAT3 with H-ras in H-ras transformed cells. Panel A: STAT3 expression in parental vs. ras-transformed NIH3T3 cells. NIH3T3 cells (lanes 1 and 2) or the ras-transformed counterpart cells (lane 3 and 4) were treated with media alone (lanes 1 and 3), or 30 ng/ml IL-3 (lanes 2 and 4) for 15 min. Total cell lysates were prepared, which were then subjected to immunoblotting using anti-STAT3 Ab. Panel B: Co-immunoprecipitation of H-ras and 91 kDa STAT3. The same cell lysates used for lanes 3 and 4 in Panel A were subjected to immunoprecipitation with anti-H-Ras Ab, followed by immunoblotting with anti-STAT3 Ab. Panel C: Interaction of phosphoserine (S727) STAT3 with H-Ras. The same lysates that were obtained from the H-ras-transformed cells used for lanes 3 and 4 in Panels A and B, were immunoprecipitated with anti-H-ras Ab and serine (S727)-STAT3 Ab. The pS-STAT3 band corresponds to the 91 kDa STAT3 that was detected in Panel B.

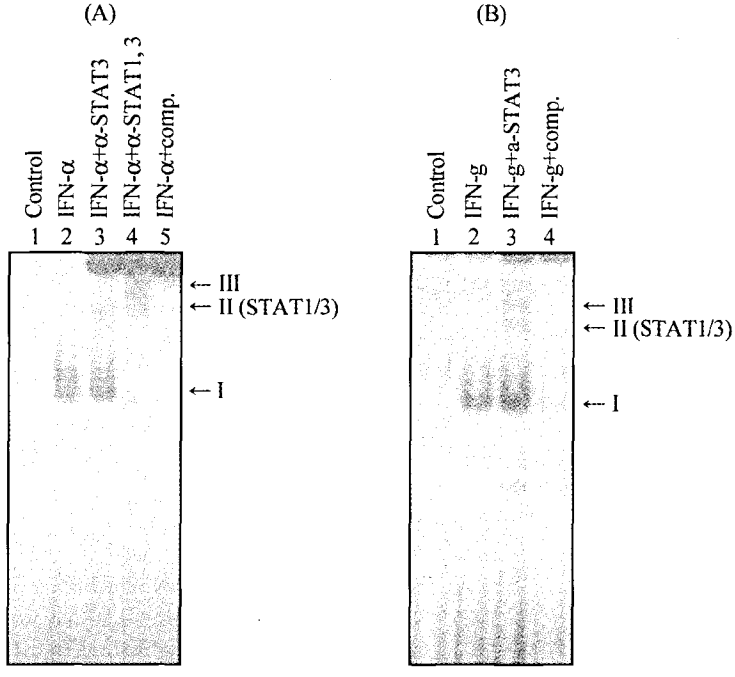

Fig. 3. Induction of STAT1/STAT3 heterodimeric complex binding to Fcγ RI GAS probe by IFN-α and IFN-γ in human tonsillar primary immune cells. Tonsillar mononuclear cells were either treated with media alone, IFN-α (5,000 U/ml), or IFN-γ (10,000 U/ml). Nuclear extracts were prepared and EMSA was performed using Fcγ RI GAS as a probe. To demonstrate the identity of the complexes, anti-STAT3 or anti-STAT1 antibodies were preincubated with the extracts for 30 min prior to the binding reaction with the labeled oligomer, as indicated. Competitor cold oligomers (100×) were also added to the binding reaction 30 min prior to the addition of the labeled oligomer.

In diverse cell types, the STAT complexes were formed primarily as homodimers. Each STAT homodimer functions as a transcriptional factor for specific target genes by binding to their promoters (Leonard and OShea, 1998). Notably, STAT3 is the first member of STATs that is involved in the formation of heterodimeric STAT complexes, STAT1/STAT3, upon stimulation of the cells with IL-2, IL-6, or IFN-α (Darnell, 1997). Thus, we were interested in examining the

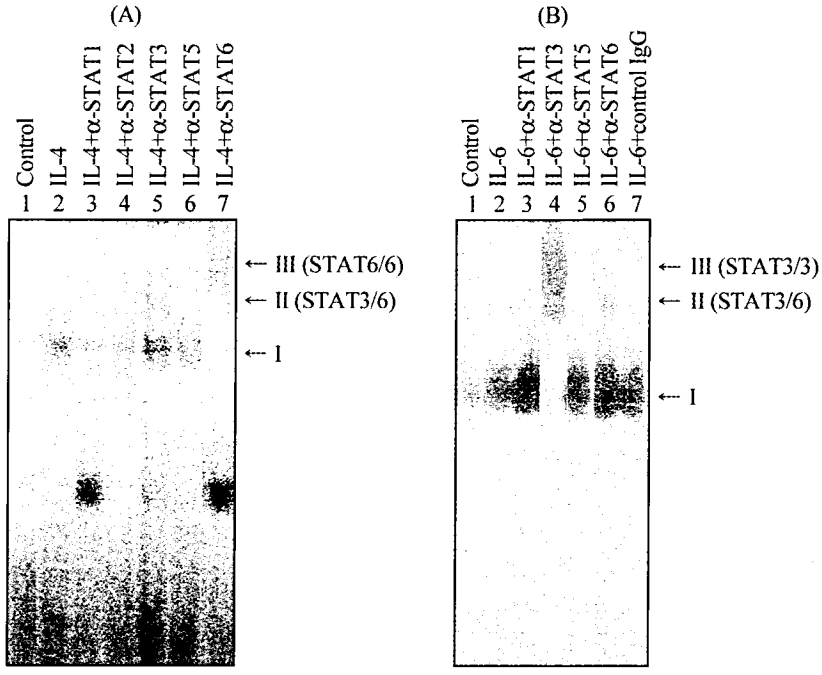

Fig. 4. Induction of STAT3/STAT6 heteromeric complex binding to Fcε RII or Fcγ RI GAS by IL-4 or IL-6. Panel A: Tonsillar mononuclear cells were treated with media alone (lane 1) or IL-4 (400 U/ml) for 15 min (lanes 2 to 7). Nuclear extracts were prepared and subjected to EMSA using the Fcε RII GAS probe. Antibodies to distinct STAT proteins were pre-incubated, and the band-supershift was analyzed. Except for anti-STAT6, only the anti-STAT3 antibody produced a partial supershift (lane 5, band II) of the major STAT6-containing complexes (band I). Panel B: Mouse myeloma SP2/0 cells were treated with media alone or IL-6 (100 U/ml) for 15 min (lanes 2 to 7). Nuclear extracts were prepared after which EMSA was performed using Fcγ RI GAS and antibodies to various STATs. The STAT6 antibody produced a partial super shift (lane 6, band II) of the major STAT3-binding complexes (band I).

ability of STAT3 to participate in transcriptional regulation by different cytokines through the formation of diverse STAT heterodimers. In primary lymphocytes, IL-4 and IFN-γ are known to selectively activate STAT6 and STAT1, respectively. Activated STATS then form unique homodimeric complexes that bind to the GAS of various target genes, whose expressions are often counter-regulated by prototypic Th2 and Th1 cytokines (Park *et al.*, 1998; So *et al.*, 2000).

Using tonsillar mononuclear cells we have observed that not only IFN-α, but also IFN-γ-induced Fcγ RI GAS-binding activities contain STAT3, in addition to the characteristic STAT1/STAT1 and STAT3/STAT3 homodimers (band I in Fig. 3-A and B). A part of these IFN-α- or IFN-γ-induced GAS binding complexes contained a component that is supershifted by the anti-STAT3 antibodies, representing a formation of the STAT1/STAT3 heterodimer (band II in Fig 3-A and B). Also, as in Fig 4-A, the IL-4-induced factors binding to the promoter of Fcε RII (Fcε RII GAS) contained a STAT3/STAT6 heterodimer (band II) in addition to the usual STAT6 homodimer (band I). Similarly, in IgE-producing myeloma cells, where IL-6 was implicated as a major growth-promoting factor (Klein *et al.*, 1987), a part of the IL-6-induced GAS-binding complexes contained a STAT3/STAT6 heterodimer, as shown by the anti-STAT6 antibody super-shift (band II, Fig. 4-B). These results suggest a possible role of STAT3/STAT6 or STAT3/STAT1 complexes in regulating the Fcε RII or Fcγ RI expression in diverse immune cells.

Considering that multiple consensus STAT-binding sites are usually present in the promoters of genes that are counter-regulated by IL-4/IL-6 (Th2 cytokine) and IFN-γ (Th1 cytokine), the ability of STAT3 to form a heterodimeric complex with STAT6 or STAT1 may present a more versatile mechanism for regulating the expression of these genes (Xu *et al.*, 1996). Interaction of STAT3 with other transcriptional factors would add another layer of complexity and versatility in the regulation of gene expressions by STAT3 (Schaefer *et al.*, 1995; Zhang *et al.*, 1997). Further studies are in progress to delineate the molecular mechanisms of STAT3 action in regulating various cytokine-induced signaling pathways by examining the functional and physical interaction of STAT3 with other signaling molecules and transcriptional factors.

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