JAK/STAT3-dependent Activation of the RalGDS/Ral Pathway in M1 Mouse Myeloid Leukemia Cells*

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The Ras-related GTPase (Ral) is converted to the GTP-bound form by Ral guanine nucleotide dissociation stimulator (RalGDS), a putative effector protein of Ras. Recently, it was proven that Ras regulates c-Src activity and subsequent phosphorylation of its substrate, STAT3. Here, we show that STAT3 inversely regulates activation of Ras through induction of expression of RalGDS. To identify new leukemia inhibitory factor-induced genes, we have performed representational difference analysis using M1 mouse myeloid leukemia cells and cloned RalGDS. The expression of RalGDS and subsequent activation of RalA were clearly suppressed by a dominant negative form of STAT3 and a JAK inhibitor, JAB/SOCS1/SSI-1, indicating that RalGDS/RalA signaling requires the activation of the JAK/STAT3 pathway. An experiment using a Ras inhibitor demonstrated that full activation of RalA also requires activation of Ras. These results suggest a novel cross-talk between JAK/STAT3 and the Ras/RalGDS/Ral signaling pathways through gp130.

Members of the interleukin-6 (IL-6) cytokine family, which include leukemia inhibitory factor (LIF), ciliary neurotrophic factor, oncostatin M, interleukin-11, and cardiotrophin-1, are involved in a variety of biological responses including the immune response, inflammation, hematopoiesis, and oncogenesis through the regulation of cell growth, survival, and differentiation. These cytokines use gp130 as a common receptor subunit. The binding of ligands to gp130 activates the JAK/STAT signal transduction pathway, where STAT3 plays a central role in transmitting the signals from the membrane to the nucleus (1). So far, a considerable number of candidate target genes of STAT3 have been characterized, most of which belong to genes regulating cell cycle progression and/or anti-apoptosis. For instance, cyclin D1/D2/D3/A, cdc25A, e-my, pin-1, bcl-2, and bcl-x are up-regulated by STAT3 activation (2–5). In contrast, in some cases, STAT3 contributes to cell cycle arrest by inducing cyclin-dependent kinase inhibitors such as p19INK4d and p21Cip1 (6, 7). Their expression is directly or indirectly controlled by STAT3. However, it is difficult to explain the molecular mechanisms of the diverse signaling effects of STAT3 by any combination of these already identified genes, further identification of STAT3-regulated genes is required.

RalGDS was initially identified as a gene homologous to the CDC25 family and found to stimulate the release of guanine nucleotide from Ras (8). A distinct family of Ras-related GTPases, Ras, consists of two highly similar proteins, RaA and RAIB (85% identical) (9). The function of Ras proteins has yet to be further investigated, but recent reports suggest their crucial role in cellular transformation, differentiation, vesicle transport, and receptor endocytosis (10–12). Recently, Goi et al. (13) demonstrated that Ras is essential for STAT3 tyrosine phosphorylation induced by epidermal growth factor or IL-6 and that Src kinase activation by Ras plays a key role in this process.

To identify the genes involved in signal transduction via gp130, we have performed representational difference analysis (RDA) using M1 cells, which differentiate into macrophages upon STAT3 activation by LIF/IL-6. Here we demonstrate that RalA is activated by the JAK/STAT3/RalGDS pathway, and its activity is also modulated by activation of Ras in M1 cells.

EXPERIMENTAL PROCEDURES

Cells and Cytokines—M1 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% horse serum. Single-cell suspensions of splenocytes were prepared from spleen of 6-week-old Std-ddY mice (Japan SLC Inc.). For cell stimulation, 15 ng/ml recombinant murine LIF (Strathmann Biotech), 50 ng/ml recombinant human IL-6 (KIRIN Brewer Co. Ltd.) or 50 ng/ml recombinant human soluble IL-6 receptor (sIL-6R) (R & D System) was used. M1/Y705F cell line was established by infecting M1 cells with retrovirus containing pMX-neo-Myc-Y705F, which was mutated by polymerase chain reaction (14). Northern Blot Analysis—0.5 μg of mRNA or 5 μg of total RNA was electrophoresed on a 1% agarose/formaldehyde gel and blotted to Hybond-N nylon filter (Amersham Pharmacia Biotech). The filter was hybridized with 32P-labeled cDNA probes in rapid hybridization buffer (Amersham Pharmacia Biotech).

Immunoblotting Analysis—Whole cell extracts were prepared by lysing cells in Laemml SAMPLE buffer. An equivalent amount of cell lysate was electrophoresed on SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membrane (Immobilon, Millipore), stained with antibodies, and detected for signals with the ECL system (Amersham Pharmacia Biotech).
measured as previously described (15, 16). M1 cells (3 x 10⁶) were lysed with the lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 1% Nonidet P-40, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) and were incubated with GST-RIP1 or GST-Raf protein bound to agarose beads for 1 h at 4 °C. The beads were subjected to SDS-gel electrophoresis followed by blotting with antibodies to either RaLA, RaLB, or Ras (Transduction Laboratory). To produce GST-RIP1 and GST-Raf, DNA fragments corresponding to amino acids 397–518 of RIP1 and 51–131 of Raf were subcloned to pGEX-4T-3 (Amersham Pharmacia Biotech), respectively.

RESULTS

cDNA libraries were prepared from M1 cells treated with LIF for 1 h and untreated M1 cells and were applied for RDA as a tester and a driver, respectively (17, 18). After three rounds of subtraction, we sequenced 85 DNA fragments. Fourteen fragments were part of the RalGDS gene, corresponding to regions 3098–5327 (GenBank™ accession number L07924). To confirm that RalGDS is induced upon LIF stimulation, we examined its expression by Northern blot analysis. RalGDS mRNA was obviously induced 1 h after LIF stimulation and down-regulated to basal level at 9 h (Fig. 1A). Stimulation with IL-6 also increased RalGDS mRNA at a similar level (Fig. 1B). While there exist other Ral-specific guanine nucleotide exchange factors (RalGEF) such as Rgl and Rlf (19, 20), the addition of CHX did not affect RalGDS expression, suggesting that RalGDS might be directly regulated by STAT3. However, PD98059 had no effect on induction of RalGDS expression. To investigate whether this process requires de novo protein synthesis, M1 cells were stimulated with 10 ng/ml of CHX for 1 h and then stimulated with LIF for 1 h. mRNA was analyzed as in C. The membrane was reprobed with the G3PDH gene (C, D), (−), non-stimulated; (+), stimulated with LIF.

To investigate whether the induction of RalGDS depends on STAT3 activation, we analyzed its induction in two cell lines: M1/Y705F that expresses a dominant-negative form of STAT3 (Tyr-705) than parental M1 cells (Fig. 2A). The induction of RalGDS mRNA was clearly reduced in both M1/Y705F and M1/JAB (Fig. 2C, lanes 4 and 6). These data strongly suggest that the induction of RalGDS is dependent on the activation of the JAK/STAT3 signaling pathway.

In addition to the JAK/STAT3 pathway, LIF/IL-6 also activates the SHP-2/Grb2/MAP kinase pathway through gp130 (2, 22). Thus, we also investigated the possibility that the induction of RalGDS might require the activity of MAP kinase. As shown in Fig. 2B, MAP kinase was activated in M1 cells upon LIF stimulation, and a MEK inhibitor, PD98059, inhibited activation. However, PD98059 had no effect on induction of RalGDS mRNA (Fig. 2C, lane 8), indicating that the SHP-2/Grb2/MAP kinase pathway is dispensable for the up-regulation of RalGDS expression. To investigate whether this process requires de novo protein synthesis, M1 cells were stimulated in the presence of cycloheximide (CHX). As shown in Fig. 2D, addition of CHX did not affect RalGDS expression, suggesting that RalGDS might be directly regulated by STAT3.

Next, we examined whether the enhanced expression of RalGDS would bring about the activation of Ral in response to LIF stimulation. Activation of Ral was evaluated by the levels of phosphorylation of tyrosine on 705 of STAT3 (Tyr-705) than parental M1 cells (Fig. 2A). Corresponding to the levels of phosphorylation of Tyr-705, the induction of RalGDS mRNA was clearly reduced in both M1/Y705F and M1/JAB (Fig. 2C, lanes 4 and 6). These data strongly suggest that the induction of RalGDS is dependent on the activation of the JAK/STAT3 signaling pathway.
Interestingly, activation of RalA was detected in neither M1/Y705F nor M1/JAB, (Fig. 3B, lanes 2 and 4). On the other hand, PD98059 had no effect on RalA activation (Fig. 3B, lane 6). These data strongly indicated that activation of RalA could be downstream of the JAK/STAT3 signaling pathway rather than via a MAP kinase pathway. In addition, a protein synthesis inhibitor, CHX, diminished the activation of RalA, which indicated that de novo protein synthesis is required for the activation of RalA (Fig. 3B, lane 8).

It has been reported that another GTPase molecule, Ras, is necessary for the activation of Ral, possibly by the recruitment of RalGDS to the cytoplasmic membrane (24–26). Thus, we examined the activity of Ras in M1 cells by in vitro binding to the GST-Raf fusion protein. Ras was already activated 0.5 h after stimulation with LIF, and the activation continued to 3 h (Fig. 4A). To examine whether Ras activity is required for full activation of RalA, we utilized FTI-277, a specific farnesylation inhibitor. After 24 h of treatment with FTI-277, M1 cells were stimulated with LIF and then subjected to the Ras or RalA activation assay. As shown in the lower panel of Fig. 4B, FTI-277 converted about 50% of Ras into a non-farnesylated form (27), followed by decreased activation of both Ras and RalA (Fig. 4B, a and b). On the other hand, FTI-277 did not suppress the induction of RalGDS (Fig. 4Bc). These results indicate that activation of RalA by LIF are also modulated by the activity of Ras. Some evidence suggests the existence of other signaling molecules such as Rap GTPases to activate the RalGDS/Ral pathway (11), but the activation of Rap1 was not observed in M1 cells (data not shown).

**DISCUSSION**

IL-6 family cytokines transduce signals into the cell by binding to their cognate receptors, followed by dimerization and activation of JAK kinases, which subsequently phosphorylate tyrosine residues of gp130 to recruit signal-transducing molecules, most notably STAT1/STAT3 and SHP-2. Tyrosine-phosphorylated STAT3 enters the nucleus, where it regulates the transcription of a variety of genes. On the other hand, SHP-2 interacts with Grb2 and Gab1 and mediates signals to ERK MAP kinase. Thus gp130 generates two distinct major signaling pathways (1).

In this study, we have demonstrated that RalGDS mRNA, one of the effector molecules of Ras (24), is clearly induced by LIF/IL-6 in M1 cells. This induction appears rather specific to RalGDS among the RalGEF because Rgl or Rlf was not induced. Moreover, we have shown that induction of RalGDS is definitely regulated by JAK/STAT3 signaling and that the activity of RalA is dependent on the transcription of RalGDS and on the gp130/Ras pathway, which is likely to involve an SHP-2/Grb2/Sos cascade (22) (Fig. 5). As far as we know, this is the first report that a small G protein is activated by transcriptional induction of a guanine nucleotide exchange factor.

The finding of an increase of RalGDS expression in IL-6-stimulated splenocytes may provide evidence for a physiological involvement of RalGDS induction in gp130 signaling. However,
its induction by STAT3 activation seemed to be cell-type specific. Whereas a murine lymphoid subline, DA1.a, expressed RalGDS mRNA upon LIF stimulation, G-CSF, which activates STAT3, did not induce its expression in a murine myeloid cell line, 32D (data not shown). These results suggest that the induction of RalGDS might require some events other than STAT3 activation.

Goi et al. (13) reported that activated Ral regulates the phosphorylation of STAT3 through activation of Src kinase. Together with their observation, our results provide the possibility that STAT3 and Ral regulate each other to exert biological functions. Indeed, some studies have shown that Ral and STAT3 exert similar biological functions. Introduction of the catalytic domain of RalGEF, Rgr, into PC12 cells inhibited NGF-induced neurite outgrowth whereas a dominant-negative catalytic domain of RalGEF, Rgr, into PC12 cells inhibited STAT3 activation. Together with their observation, our results provide the possibility that STAT3 and Ral regulate each other to exert biological functions. Indeed, some studies have shown that Ral and STAT3 exert similar biological functions. Introduction of the catalytic domain of RalGEF, Rgr, into PC12 cells inhibited NGF-induced neurite outgrowth whereas a dominant-negative catalytic domain of RalGEF, Rgr, into PC12 cells inhibited STAT3 activation. These results suggest that the induction of RalGDS might require some events other than STAT3 activation.

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