Conditional Activation of Janus Kinase (JAK) Confers Factor Independence upon Interleukin-3-dependent Cells

ESSENTIAL ROLE OF Ras IN JAK-TRIGGERED MITOGENESIS*

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Cytokines play crucial roles in the growth and differentiation of hematopoietic cells. They bind to specific cell membrane receptors that usually do not possess intrinsic protein-tyrosine kinase activity. Janus kinases (JAKs) are cytoplasmic protein-tyrosine kinases that physically interact with intracellular domains of the cytokine receptors and have been implicated in playing important roles in signal transduction triggered by the cytokine-cytokine receptor interaction. However, it is still uncertain whether JAK activation alone suffices to induce cell proliferation. In this work, we modified Tyk2, a member of the JAK family, by adding a membrane localization sequence and a chemical dimerizer (coumeyrmycin)-dependent dimerization sequence. The modified Tyk2 was activated in a coumeyrmycin-dependent manner, and the activated Tyk2 conferred cytokine independence upon interleukin-3-dependent pro-B lymphoid cells. This cytokine-independent proliferation was completely inhibited by dominant-negative Ras. These results indicate that activation of JAK through membrane-proximal dimerization is sufficient to induce cell cycle progression and that Ras is essentially involved in JAK-triggered mitogenesis.

Growth and differentiation of hematopoietic cells are tightly controlled by a distinct class of humoral factors termed cytokines (1). The spatial and temporal expression of particular sets of cytokines ensures the propagation and differentiation of specialized lineages of hematopoietic cells. Cytokines interact with specific cell membrane receptors that, in general, do not possess intrinsic protein-tyrosine kinase activity (2, 3). However, recent studies have shown that the cytokine receptors associate with cytoplasmic nonreceptor-type protein-tyrosine kinases, thereby forming molecular complexes that are functionally equivalent to the receptor-type protein-tyrosine kinases. Of these kinases, Janus kinases (JAKs) have been implicated as playing crucial roles in cytokine signaling because they physically bind to the receptor cytoplasmic regions that appear to be critical in mitogenic signal transduction, and their kinase activity is rapidly stimulated by cytokine binding to these receptors (4). Activated JAKs phosphorylate a series of substrates that include a class of transcription factors termed STATs. To date, seven different STATs have been described. Once phosphorylated, the STATs translocate from the cytoplasm to the nucleus, where they initiate transcription of cytokine-responsive genes (5, 6). Different cytokine receptors activate distinct sets of JAKs and STATs upon ligand stimulation, and this may explain, at least in part, the redundant and pleiotropic actions of cytokines.

Cytokines are known to activate multiple cytoplasmic signal-transducing molecules in addition to JAKs. Src family proteins are another class of cytoplasmic protein-tyrosine kinases that are rapidly activated in response to a variety of cytokine signals (7–11). Ras signaling pathways are also activated by cytokines; a variety of cytokines induce tyrosine phosphorylation of Shc (12, 13), an increased GTP-bound form of Ras (14, 15), activation of Raf-1 (16, 17), and activation of mitogen-activated protein kinase cascades (18, 19). Cytokine signals also activate phosphatidylinositol 3-kinase (20). However, the precise roles of these cytoplasmic signal transducers in cytokine-mediated cell growth and differentiation are not well understood.

Concomitant activation of multiple signaling pathways that lie downstream of the cytokine receptors makes it difficult to pursue specific roles of JAKs in cytokine-triggered mitogenesis. Receptor reconstitution studies have shown that mutant cytokine receptors that cannot interact with JAKs fail to transmit mitogenic signals (21–25). Although the observations indicate important roles for JAKs in cell proliferation, they do not exclude the possibility that other signal transducers interact with the same regions that JAKs bind, or alternatively, such receptor mutations may disrupt structural integrity of the intracellular domains and inactivate receptor function.

Recently, a chimeric molecule consisting of the extracellular epidermal growth factor receptor and the cytoplasmic Jak2 kinase domain was shown to confer epidermal growth factor dependence upon interleukin-3 (IL-3)-dependent cells (26). Furthermore, a fusion protein consisting of the catalytic domain of Jak2 and TEL, a member of the ETS transcription factor family, transformed IL-3-dependent cells into factor independence (27). The results indicate that the catalytic domain of JAK is capable of generating sufficient inputs to promote cell proliferation. In contrast, simple overexpression of wild-type Jak2 or a hyperactive form of Jak2 failed to convert cytokine-dependent cells to factor independence (28).

In this work, we demonstrate that IL-3-dependent cells acquire cytokine independence by a conditional, membrane-proximal activation of Tyk2, a member of the JAK family, with the use of a recently developed chemical dimerization technique (29, 30). The establishment of the conditional Tyk2-dependent cells made it possible to study downstream elements of JAK in...
mitogenesis in the absence of concomitant activation of other signaling pathways that lie downstream of the cytokine receptors. We provide evidence that Ras is critically involved in JAK-dependent mitogenesis.

MATERIALS AND METHODS

Cells—6-1 cells were made from the Ba/F3-derived mouse pro-B cell line F7 (31) by stably cotransfecting expression vectors for the tetracycline-repressible transactivator (32) and the bacterial lac repressor. The 6-1 cells were cultured in cytokine-free medium for 30 h, and luciferase activities were measured. cDNA expression was suppressed by tetracycline and induced by IPTG in the same batch of transfected cells. Data were normalized for percent induction of those stimulated by IL-3 for the last 6 h. Data shown are representative of three independent experiments. B, 6-1 cells were transiently cotransfected with the c-fos promoter-luciferase reporter plasmid and an inducible expression plasmid for wild-type Tyk2 (WT-Tyk2) or its kinase-dead mutant (KN-Tyk2). Relative luciferase activities were determined as described for A.

Fig. 2. Activation of endogenous Tyk2 by IL-3 in 6-1 cells. The 6-1 cells were growth-arrested by culturing in cytokine-free medium. After 8 h, cells were re-stimulated with IL-3 and harvested at the indicated times after stimulation. Whole cell lysates were prepared from the cells and subjected to immunoprecipitation with anti-Tyk2 antibody. The immunoprecipitates (I.P.) were analyzed by immunoblotting with anti-phosphotyrosine antibody (α-PY; upper). The same filter was re-probed with anti-Tyk2 antibody (lower).

Fig. 3. Artificial membrane localization and dimerization of Tyk2 in 6-1 cells. A, schematic of the modified Tyk2 constructs. G-Tyk2 consists of the B subunit of bacterial DNA gyrase (shaded box) and human Tyk2 lacking the amino-terminal 25 amino acids. SG-Tyk2 was made by adding the myristoylation signal sequence of v-Src (striped circle) at the amino terminus of G-Tyk2. WT-Tyk2, wild-type Tyk2. B, c-fos promoter activation by the modified Tyk2. The 6-1 cells were transiently transfected with the c-fos promoter-luciferase reporter plasmid and an inducible expression plasmid for G-Tyk2 or SG-Tyk2. Cells were cultured in cytokine-free medium for 30 h. G-Tyk2 or SG-Tyk2 was conditionally dimerized by adding coumermycin to the culture. Relative luciferase activities were determined as described for Fig. 1.
RESULTS

Activation of Proliferation-associated Genes by JAKs—To investigate the potential role of JAKs in cell proliferation, we first addressed the ability of each member of the JAK family (Jak1, Jak2, Jak3, and Tyk2) to activate the expression of genes involved in G1/S, cell cycle progression by transiently expressing each in the Ba/F3-derived subline 6-1 (31). The cDNA was inducibly expressed in the IL-3-dependent 6-1 cells using a modified tetracycline-repressible promoter (TcIP promoter) (32, 41) whose activity was suppressed by tetracycline and induced by the lactose analog IPTG.

Reporter plasmids were chosen to serve as indicators of proliferation-associated genes. They were constructed by connecting the luciferase gene downstream of the c-fos, c-myc, and cyclin D3 promoter sequences, respectively. These promoters were all activated upon IL-3 stimulation in 6-1 cells. Transient expression of each JAK except Jak3 in the 6-1 cells resulted in cDNA, a synthetic oligonucleotide encoding the myristoylation signal sequence of v-Src (33) was ligated in frame to the 5'-end of G-Tyk2 cDNA. G-Tyk2 and SG-Tyk2 cDNAs were then cloned into pOPTET-BSD. A cDNA encoding c-Ha-Ras<sup>177</sup> was cloned into pOPTET-puro, which possesses the puromycin resistance gene instead of the BSD gene. Reporter plasmids were constructed from the pGL3-Basic vector by connecting the luciferase gene downstream of the c-fos, c-myc, and cyclin D3 promoter sequences, respectively.

Luciferase Assay—The 6-1 cells were transiently transfected with 1.25 μg of luciferase reporter plasmid and 2.5 μg of expression plasmid vector by the DEAE-dextran method. The same batch of transfected cells were split and cultured in cytokine-free medium for 30 h in the presence of either 1 μg/ml tetracycline or 5 mM IPTG before the luciferase assay. For conditional dimerization of the modified Tyk2, cells were treated with 300 nm coumermycin. Luciferase activities were measured by the luciferase assay kit (Promega) according to the manufacturer’s instructions.

Stable Transformation and Screening of the Cytokine-independent Cell Line—The expression plasmid (2–10 μg) was linearized and transfected into 1 × 10<sup>6</sup> 6-1 cells by electroporation. Transfected cells were cultured in RMPI 1640 medium supplemented with 10% fetal calf serum and 20% WEHI-3B-conditioned medium in the presence of either 1 μg/ml tetracycline or 5 mM IPTG for 30 h before the luciferase assay. For establishing cytokine-independent cell lines, bacteria s-resistant cells obtained by transfecting plasmid pOPTETBSD-SG-Tyk2 were collected, washed, and cultured in cytokine-free RMPI 1640 medium supplemented with 10% fetal calf serum in the presence of 5 mM IPTG and 300 μM coumermycin.

Immunoprecipitation and Immunoblotting—Proteins were induced in the stable transfecteds with 5 mM IPTG and extracted with ELB buffer (34). For sequential immunoprecipitation/immunoblotting, cell lysates were first treated with anti-Tyk2 or anti-Stat5 antibody for 3 h. The immune complexes were then collected with protein A-Sepharose. Total cell lysates or immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride filters, and immunoblotted with appropriate antibodies. Proteins were visualized using an enhanced chemiluminescence detection system (NEN Life Science Products). Antibodies used were anti-Tyk2 (Santa Cruz sc-189), anti-Stat5 (Santa Cruz sc-835), anti-phosphotyrosine (4G10; Upstate Biotechnology 05-321), anti-Raf-1 (Santa Cruz sc-133), and anti-Ha-Ras (Santa Cruz sc-520).

RESULTS


growth-arrested by culturing in cytokine-free medium. After 8 h, cells were re-stimulated with IL-3 and harvested at the indicated times after stimulation. Whole cell lysates were prepared from the cells and subjected to anti-Raf-1 immunoblotting. The slower mobility of Raf-1 seen after stimulation with IL-3 reflects the increased phosphorylation associated with the activation of Raf-1 kinase.

Fig. 4. Transformation of 6-1 cells by conditionally activated Tyk2. A, expression of SG-Tyk2 in the cytokine-independent transfectants. Upper, total cell lysates prepared from the two representative SG-Tyk2 transfectant clones (SG-0-1 and SG-15-2) cultured in the presence of either tetracycline (−induction) or IPTG (+ induction) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-Tyk2 antibody. Lower, growth of SG-15-2 cells was examined by culturing them in cytokine-free medium containing tetracycline (−Tyk2 induction) or IPTG (+ Tyk2 induction) in the absence (−Tyk2 dimerization) or presence (+ Tyk2 dimerization) of coumermycin. Viable cell number was counted at 24-h intervals by trypsin blue dye exclusion methods. B, coumermycin-dependent autophosphorylation of SG-Tyk2. Cells were growth-arrested in cytokine-free medium containing IPTG in the absence of coumermycin for 18 h. The growth-arrested cells were then treated with coumermycin, and the cell lysates were prepared at the indicated time points. The lysates were immunoprecipitated with anti-Tyk2 antibody, and the immunoprecipitates were then subjected to SDS-polyacrylamide gel electrophoresis and subjected to anti-phosphotyrosine immunoblotting. C, coumermycin-dependent tyrosine phosphorylation of Stat5. The SG-Tyk2 transfectants were cultured in cytokine-free medium containing tetracycline (−Tyk2 induction) or IPTG (+ Tyk2 induction) in the absence (−Tyk2 dimerization) or presence (+ Tyk2 dimerization) of coumermycin for 24 h and were lysed. The cell lysates were immunoprecipitated with anti-Tyk2 (left) or anti-Stat5 (right) antibody. The immunoprecipitates (I.P.) were then subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-phosphotyrosine antibody (−PY, upper). The filters were re-probed with anti-Tyk2 or anti-Stat5 antibody (lower).
FIG. 6. Effect of Ras<sup>N17</sup> on Tyk2-dependent mitogenesis. A, inhibition of Tyk2-dependent activation of proliferation-associated genes by Ras<sup>N17</sup>. The 6-1 cells were triply transfected with a luciferase reporter plasmid, an inducible expression plasmid for SG-Tyk2, and an inducible expression plasmid for Ras<sup>N17</sup>. Luciferase assays were performed as described for Figs. 1 and 3. B, establishment of SG-Tyk2/Ras<sup>N17</sup> doubly stable transfectants. Left, induction of Ras<sup>N17</sup> by IPTG in two representative doubly stable transfectants (SG/dnR-2-8 and SG/dnR-3-7) was examined by anti-c-Ha-Ras immunoblotting. Right, IL-3-dependent growth of SG/dnR-2-8 cells that conditionally overexpress Ras<sup>N17</sup> was examined by culturing...
a significant activation of these promoters in the absence of IL-3 (Fig. 1A). Among the JAKs examined, Tyk2 provoked the strongest activation of all three promoters. The promoter activation required an active kinase because a kinase-dead Tyk2 mutant completely lacked this activity (Fig. 1B). It is unknown whether differences in promoter activation among different JAKs reflect levels of protein induced in the transiently transfected cells or specific roles of these JAKs in gene regulation. Notably, Tyk2 was expressed in the 6-1 cells and was activated in response to IL-3 as determined by its autophosphorylation status (Fig. 2).

### Generation and Properties of Conditionally Dimerizable Tyk2—JAKs interact with the cytoplasmic domains of the cytokine receptors, and upon ligand-induced oligomerization of the receptor, the receptor-associated JAKs may become juxtaposed and transphosphorylate each other to be activated. Hence, we suspected that artificial membrane-proximal localization and dimerization of JAK could mimic a cytokine-activated form of JAKs. Accordingly, we used Tyk2 as a representative of the JAKs, modifying it so that it could be conditionally dimerized. To do so, we employed a recently developed chemical dimerization technique (29, 30). The amino-terminal subdomain (219 amino acids) of the B subunit of bacterial DNA gyrase (GyrB) homodimerizes in the presence of a chemical dimerizer, coumermycin. Hence, we connected GyrB to the amino-terminal region of Tyk2 to generate a Tyk2 mutant (G-Tyk2) that homodimerizes in the presence of coumermycin. We also introduced the myristoylation sequence (14 amino acids) of v-Src into the amino terminus of G-Tyk2, expecting to dimerize Tyk2 in the presence of coumermycin. As a control, we generated G-Tyk2 that homodimerizes in the presence of coumermycin. We also introduced the myristoylation sequence (14 amino acids) of v-Src into the amino terminus of G-Tyk2, expecting to target G-Tyk2 at the inner surface of plasma membrane as has been reported previously (33). The resultant construct was termed SG-Tyk2 (Fig. 3A).

The cDNA encoding G-Tyk2 or SG-Tyk2 was inserted downstream of the TcIP promoter and transiently introduced together with the c-fos promoter-luciferase reporter plasmid into 6-1 cells, and the effect of the modified Tyk2 on c-fos promoter was examined (Fig. 3B). Induction of G-Tyk2 by IPTG led to a significant c-fos promoter activation that was further potentiated upon coumermycin treatment, indicating that dimerization of Tyk2 augmented c-fos gene activation. Expression of SG-Tyk2 resulted in stronger c-fos promoter activation than that induced by G-Tyk2 alone in the absence of coumermycin. Furthermore, dimerization of SG-Tyk2 by coumermycin resulted in a synergistic effect on the activation of the c-fos promoter. These results indicate that membrane tethering and dimerization of Tyk2 are two critical processes underlying the activation of the c-fos promoter by Tyk2.

**Transformation of IL-3-dependent Cells into Factor Independence by the Modified Tyk2**—The above observations suggested that ectopic expression of the modified Tyk2 in the 6-1 cells could provoke certain biological effects in a coumermycin-dependent fashion. To address this possibility, we stably transferred the cDNA encoding SG-Tyk2 under the control of the TcIP promoter (pOPTET-BSD-SG-Tyk2) into 6-1 cells and selected blasticidin S-resistant cells. The cells were then pooled and cultured in cytokine-free medium in the presence of IPTG and coumermycin, which we expected to permit the constitutive expression of dimerized SG-Tyk2. After a few days of culture, a number of the drug-resistant cells started to proliferate despite the absence of IL-3. These cytokine-independent cells were then single cell-cloned by limiting dilution. All the independently established clones constitutively expressed high levels of SG-Tyk2 that could not be efficiently suppressed by tetracycline (Fig. 4A, upper). This was most likely due to the growth advantage of the high SG-Tyk2 expressors selected under the cytokine-free culture conditions. However, the cytokine-independent proliferation was completely dependent on coumermycin because its withdrawal from the culture resulted in rapid cell growth arrest and cell death (Fig. 4A, lower). This indicated that simple overexpression of SG-Tyk2 was not sufficient to transform 6-1 cells into factor independence. The proliferation rate of cells supported by IPTG and coumermycin was comparable to that supported by IL-3.

To investigate whether the kinase activity of SG-Tyk2 in these stable transfectants was specifically regulated by coumermycin, we examined the autophosphorylation status of the SG-Tyk2 molecules. Treatment of the transfectant cells with coumermycin induced rapid and strong tyrosine phosphorylation of SG-Tyk2, indicating that the kinase was activated through the coumermycin-mediated homodimerization (Fig. 4B). Furthermore, the coumermycin treatment provoked strong tyrosine phosphorylation of endogenous Stat5 (Fig. 4C). We concluded from these observations that the kinase activity of SG-Tyk2 was strictly under the control of the chemical dimerizer coumermycin. Notably, we could not detect tyrosine phosphorylation of STATs other than Stat5 by activated SG-Tyk2, although some are relatively abundantly expressed in 6-1 cells (data not shown). This indicates that Stat5 is a major if not the sole target of SG-Tyk2 among STATs in 6-1 cells.

**Effect of Dominant-negative Ras on Tyk2-dependent Cell Proliferation**—By employing the SG-Tyk2 transfectants, we next addressed cytoplasmic signaling molecules that lie downstream of Tyk2. In particular, we wished to determine whether JAK-triggered mitogenesis involves Ras, a critical transducer of mitogenic signals triggered by receptor-type protein-tyrosine kinases. Indeed, treatment of the 6-1 cells with IL-3 activated Raf-1, a critical downstream effector of Ras, indicating involvement of the Ras pathway in IL-3-triggered mitogenesis (Fig. 5A).

Transient expression of dominant-negative c-Ha-Ras (RasN17) in the 6-1 cells strongly inhibited Tyk2-dependent induction of the c-fos, c-myc, and cyclin D3 genes as examined by a luciferase reporter assay (Fig. 6A). Accordingly, we generated doubly stable transfectants that coexpress SG-Tyk2 and RasN17 by transfecting pOPTET-puro-RasN17 into the SG-Tyk2 transfectant SG-15-2 (Fig. 6B, left). Induction of superphysiological amounts of RasN17 in the doubly stable line SG/dnR-2-8 caused little inhibition of IL-3-dependent growth (Fig. 6B, right). Hence, RasN17 overexpression itself was not toxic and was tolerated by the cells. The parental (SG-15-2) and transfectant (SG/dnR-2-8) cells were then treated with coumermycin and IPTG in cytokine-free medium to switch their growth from the IL-3-dependent state to the Tyk2-dependent state. As shown in Fig. 6C (upper), both cell types expressed comparable levels of the SG-Tyk2 molecules that were activated and phosphorylated endogenous Stat5 upon coumermycin-mediated

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**Cells in medium containing IL-3 (20% WEHI-3B-conditioned medium) in the presence of tetracycline (−RasN17 induction) or IPTG (+RasN17 induction). C, effect of dominant-negative RasN17 on Tyk2-dependent cell growth. Upper, parental SG-15-2 cells and SG/dnR-2-8 doubly stable transfectant cells were cultured in cytokine-free medium containing tetracycline (−RasN17/Tyk2 induction) or IPTG (+RasN17/Tyk2 induction) in the absence (−Tyk2 dimerization) or presence (+Tyk2 dimerization) of coumermycin, and the cell lysates were immunoprecipitated with anti-Tyk2 or anti-Stat5 antibody. Phosphorylation of Stat5 was examined by anti-phosphotyrosine (α-PY) immunoblotting of the anti-Stat5 immunoprecipitates. I.P., immunoprecipitation; W.B., Western blotting. Lower, the Tyk2-dependent growth of SG/dnR-2-8 cells that conditionally overexpress RasN17 was examined by culturing cells in cytokine-free medium containing tetracycline (−RasN17/Tyk2 induction) or IPTG (+RasN17/Tyk2 induction) in the absence (−Tyk2 dimerization) or presence (+Tyk2 dimerization) of coumermycin.**
mitogenesis. Dominant-negative c-Ha-Ras (RasN17), which is known as a potent activator of other signaling pathways that lie downstream of the cytokine receptors. Because it is a well-known model system for understanding cell cycle progression in response to cytokine. Indeed, dominant-negative Ras, which is ineffective in the IL-3-dependent growth of Ba/F3 cells, was shown to inhibit the IL-3-dependent growth of 32D myeloid cells (40). This suggests that the relative contribution of the JAK-dependent and -independent mitogenic signaling pathways varies among different cell types as well as different cytokines. The presence of two (or more) distinct signaling pathways, each of which is capable of supporting cell cycle progression in a single cell, may well explain why no cytoplasmic signal transducer has yet been found to be indispensable for cytokine-triggered cell proliferation. Molecular elucidation of components constituting the JAK-dependent and -independent pathways in cytokine-dependent mitogenesis will be a major challenge, and the conditional Tyk2-dependent cells established here should provide a powerful tool to address this issue.

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