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Ras and Signal Transducer and Activator of Transcription (STAT) Are Essential and Sufficient Downstream Components of *Janus* Kinases in Cell Proliferation

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Cytokines exert their activities in cell growth and differentiation by binding specific cell membrane receptors. *Janus* kinases (JAKs) are cytoplasmic protein tyrosine kinases that physically interact with intracellular domains of the cytokine receptors and they play crucial roles in transducing signals triggered by the cytokine-receptor interaction. We have previously shown that conditional activation of JAK through membrane-proximal dimerization confers cytokine-independence on interleukin-3 (IL-3)-dependent Ba/F3 lymphoid cells and that the cytokine-independent proliferation is completely inhibited by dominant negative Ras. In this work, we demonstrate that ectopic expression of a dominant negative form of Stat5, a major signal transducer and activator of transcription (STAT) expressed in Ba/F3 cells, also inhibits JAK-triggered mitogenesis. In contrast, overexpression of constitutively active Ras or conditional activation of Stat5 by chemical dimerization fails to confer cytokine-independence. However, concomitant activation of ectopic Ras and Stat5 molecules in Ba/F3 cells suffices for cell proliferation in the absence of IL-3. Our results indicate that Ras and STAT are essential and sufficient components of JAK-triggered mitogenesis. Our findings further indicate that the cytokine signal bifurcates into Ras and STAT pathways following JAK activation.

Key words: Cytokine — JAK — Ras — STAT — Chemical dimerizer

The growth and differentiation of hematopoietic cells are regulated by a set of humoral factors termed cytokines.[1)] Cytokines interact with homologous cell membrane receptors that in general do not possess protein tyrosine kinase activity.[2, 3)] Recent studies have shown that *Janus* kinases (JAKs) play crucial roles in cytokine signaling. JAK family proteins, termed Jak1, Jak2, Jak3 and Tyk2, are cytoplasmic protein tyrosine kinases that bind the receptor cytoplasmic regions, and their kinase activity is rapidly stimulated by cytokine treatment.[4)] Upon activation, JAKs phosphorylate substrates that include a class of transcriptional factors termed STATs (signal transducers and activator of transcription). The phosphorylated STATs translocate from the cytoplasm to the nucleus, where they initiate the transcription of cytokine-responsive genes.[5, 6)]

Cytokine-receptor interaction activates multiple cytoplasmic signaling molecules such as Src family protein tyrosine kinases, Ras and phosphatidylinositol (PI)-3 kinase in addition to the JAKs.[7–12)] Concomitant activation of a variety of signal transducers that lie downstream of the cytokine receptors makes it difficult to determine the specific roles of JAKs in cytokine-triggered cell proliferation. To overcome this problem, we modified Tyk2, one of the JAKs, so that it could mimic the receptor-associated JAK by adding a membrane-targeting signal and a chemical dimerizer-dependent dimerization domain.[13)] The modified Tyk2 transformed the interleukin (IL)-3-dependent lymphoid Ba/F3 cell into cytokine-independence in a chemical dimerizer-dependent manner.[13, 14)]

The establishment of the conditional JAK-dependent cells made it possible to study downstream elements of JAKs in mitogenesis without activating other signaling pathways. Indeed we have found that JAK-triggered mitogenesis was inhibited by ectopic expression of dominant-negative Ras.[13)] In this work, we demonstrate that, as is the case with dominant-negative Ras, dominant-negative STAT inhibits JAK-mediated proliferation of Ba/F3 cells. Conversely, concomitant activation of ectopic Ras and STAT molecules confers factor independence upon Ba/F3 cells. Our results indicate that Ras and STAT are essential and sufficient downstream components of JAKs in cytokine-dependent cell proliferation.

MATERIALS AND METHODS

Plasmid construction pOPTET-puro and pOPTET-BSD are inducible cDNA expression vectors that possess the TcIP promoter, a chimeric promoter consisting of the tetracycline-regulated promoter and the *lac*-operator.[15)] The promoter activity is negatively and positively regulated by tetracycline and isopropyl thiogalactopyranoside (IPTG),

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respectively. A cDNA encoding constitutively active c-Ha-Ras^{V12} was cloned into pOPTET-BSD. A cDNA encoding a dominant negative Stat5 (ΔStat5) was generated by amplifying a cDNA fragment corresponding to the residues 1 to 682 of Stat5b from a mouse T cell cDNA library through polymerase chain reaction and was cloned into pOPTET-puro. A dimerizable Stat5 (Stat5G) was made by ligating a cDNA encoding the dimerization domain derived from bacterial gyrase B in-frame to the 3′ end of human Stat5a cDNA and was cloned into pOPTET-puro. A reporter plasmid was constructed from pGL3-basic vector by connecting the luciferase gene downstream of the Stat5-responsive, *β-casein* promoter sequences.

Cells Ba/F3 is an IL-3-dependent mouse pro-B lymphoid cell line, and 6-1 is a Ba/F3-derived, stable transfectant that constitutively expresses the tetracycline-repressible transactivator and the bacterial *lac* repressor.[15, 16] Cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) and 20% WEHI-3B-conditioned media (20% WEHI) as a source of IL-3.

Generation of stable transfectants Stable transfectants were made from 6-1 cells or SG-15-2 cells, a 6-1-derived transfectant that conditionally expresses a dimerizable Tyk2 (SG-Tyk2),[13] by the electroporation method as described previously.[15] Transfected cells were selected in RPMI1640 supplemented with 10% FCS and 20% WEHI in the presence of 1 μg/ml tetracycline and 1.5 μg/ml puromycin (for pOPTET-puro) or 20 μg/ml blasticidin-S (for pOPTET-BSD). Stable transfectants were single-cell cloned by limiting dilution.

Luciferase assay Luciferase assay was performed according to the method described previously.[13] For conditional dimerization of Stat5G, cells were treated with 300 n*M* courmermycin.

Immunoprecipitation and immunoblotting Proteins were induced in the stable transfectants with 5 m*M* IPTG and were extracted with E1A lysis buffer (ELB).[16] For sequential immunoprecipitation-immunoblotting, cell lysates were first treated with anti-Tyk2 or anti-Stat5 for 3 h. The immune complexes were then collected on protein A-Sepharose beads. Total cell lysates or immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinyldenedifluoride filters and immunoblotted with appropriate antibodies. Proteins were visualized using the enhanced chemiluminescence detection system (NEN). Antibodies used were anti-Tyk2 (#sc-169; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Stat5 (#sc-835; Santa Cruz Biotechnology), anti-phosphotyrosine (4G10; Upstate Biotechnology), and anti-Ha-Ras (#sc-520; Santa Cruz Biotechnology).

Cell growth curve Cells were washed with phosphate-buffered saline to remove tetracycline and then resuspended at a density of 1×10^5/ml in RPMI1640/10% FCS containing either 1 μg/ml tetracycline or 5 m*M* IPTG in the absence of IL-3. For dimerization of Stat5G, courmermycin was added to the culture at the final concentration of 300 n*M*. Viable cell numbers were determined by the trypan blue-dye exclusion assay.

RESULTS

The effect of constitutively active Ras on JAK-mediated cell proliferation In our previous work, we demonstrated that dominant negative c-Ha-Ras, Ras^{N17} (serine to asparagine mutation at amino acid residue 17), effectively inhibits Tyk2-dependent proliferation of Ba/F3 cells, indicating an essential requirement of Ras activation in JAK-mediated mitogenesis.[13] Given this, we decided to address whether constitutive activation of Ras is sufficient to transform Ba/F3 cells so that they have factor-independence. To do so, we generated a conditional expression vector for the constitutively active human c-Ha-Ras,

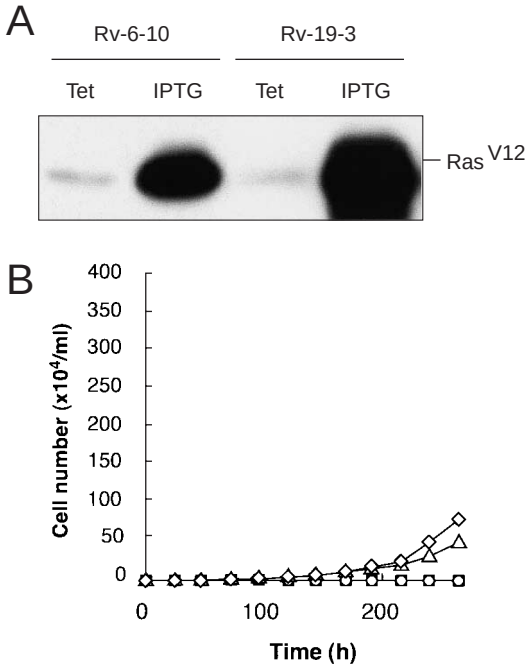

Fig. 1. Ectopic expression of constitutively active Ras in Ba/F3 cells. (A) Inducible expression of Ras^{V12} in Ba/F3-derived stable transfectants, Rv-6-10 and Rv-19-3. Total cell lysates prepared from transfectants cultured in the presence of either tetracycline (Tet) or IPTG were subjected to SDS-PAGE and immunoblotted with anti-Ras antibody. The position of Ras^{V12} is indicated. (B) The role of Ras^{V12} in Ba/F3 cell proliferation. Growth of Rv-6-10 cells was examined by culturing them in cytokine-free medium containing Tet or IPTG in the absence or presence of courmermycin (CM). □ Tet, △ IPTG, ○ Tet+CM, ◇ IPTG+CM.

Ras^{V12} (glycine to valine mutation at the amino acid residue 12), in which the cDNA expression is inhibited by tetracycline (Tet) and is potently induced by IPTG (pOPTET-BSD-Ras^{V12}). Stable transfectant clones were then generated by transfecting pOPTET-BSD-Ras^{V12} into Ba/F3-derived 6-1 cells. As shown in Fig. 1A, the obtained transfectant clones expressed ectopic Ras^{V12} in a Tc-IPTG regulated fashion.

The effect of ectopic Ras^{V12} on cell proliferation was examined by culturing the transfectants in IL-3-free medium. In the absence of IL-3, parental 6-1 cells died within 24 h. Similarly, under a non-induced condition, Ras^{V12} transfectant cells were dead within 24 h in IL-3-free medium. In contrast, cells expressing Ras^{V12} did not show any sign of apoptosis by 4 days in culture. It should be noted that the anti-apoptotic effect of active Ras in cytokine-dependent cells has already been reported.[17] To our surprise, the number of Ras^{V12}-expressing cells gradually increased after 4 days in culture, although their growth was much slower than that induced by conditional JAK activation or IL-3 treatment. Similar results were reproduced in another clone, Rv-19-3 (data not shown). This indicates that constitutively active Ras expressed in Ba/F3 cells prevents apoptosis and induces extremely weak mitogenesis in the absence of a cytokine. Furthermore, the presence of a latent time for growth indicates that epigenetic adaptation or cellular conditioning may be required to initiate cell cycle advance in Ras^{V12}-overexpressing cells. Obviously, however, activation of Ras *per se* cannot fully reproduce IL-3- or JAK-mediated cell proliferation.

The effect of dominant-negative Stat5 on JAK-mediated mitogenesis The above observation raised the possibility that effective proliferation of Ba/F3 cells requires activation of molecule(s) other than Ras. Since Stat5 is a major STAT species expressed in Ba/F3 cells and is phosphorylated/activated by JAKs,[18–20] we wondered if Stat5 is involved in JAK-dependent proliferation of Ba/F3 cells. To examine this, we generated a cDNA encoding the dominant-negative Stat5 (ΔStat5) and examined its effect on the JAK-mediated mitogenesis.[21] The ΔStat5 cDNA was inserted into the inducible pOPTET-puro vector, and was stably introduced into SG-15-2 cells, a Ba/F3-derived transfectant that conditionally expresses dimerizable SG-Tyk2 and hence proliferates in the presence of the chemical dimerizer, courmermycin (CM), in the absence of IL-3.[13] As shown in Fig. 2, inducible expression of ΔStat5 in the SG/dSt-5-5 cells (Fig. 2A) gave rise to strong inhibition of Tyk2-dependent phosphorylation of endogenous Stat5 (Fig. 2B), indicating that the dominant negative mutant worked in an induction-dependent fashion in the cells. Furthermore, upon ΔStat5 induction, the transfectant failed to proliferate, despite the activation of Tyk2 by chemical dimerization (Fig. 2C). We concluded from these

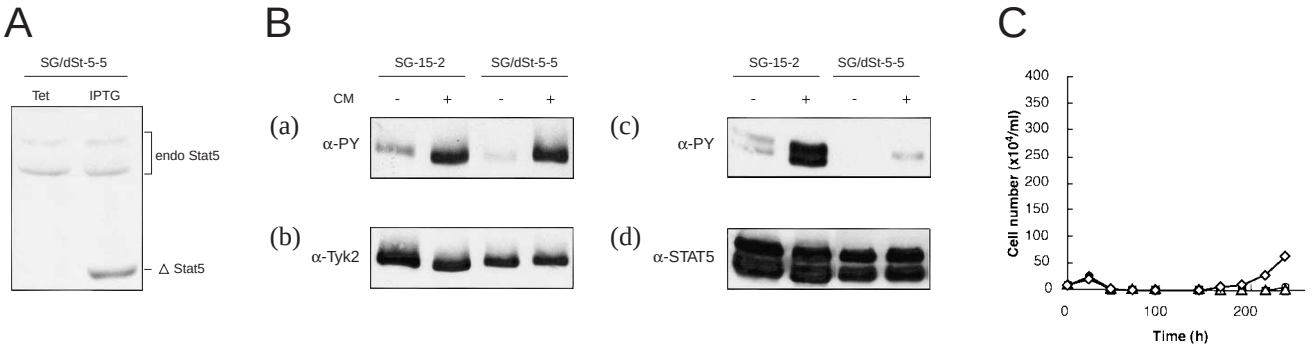

Fig. 2. The effect of dominant-negative Stat5 on Tyk2-triggered mitogenesis. (A) Inducible expression of dominant-negative Stat5 (ΔStat5) in Ba/F3-derived, conditional Tyk2-dependent cells, SG-15-2. Total cell lysates prepared from the ΔStat5 transfectant, SG/dSt-5-5, cultured in the presence of either tetracycline (Tet) or IPTG were subjected to SDS-PAGE and immunoblotted with anti-Stat5 antibody. The positions of ΔStat5 and endogenous Stat5 (endo Stat5) are indicated. (B) The effect of ΔStat5 on Tyk2-dependent phosphorylation of endogenous Stat5. Parental SG-15-2 cells and the ΔStat5-transfectant, SG/dSt-5-5, were cultured in IL-3-free medium containing IPTG to induce the conditionally dimerizable Tyk2, SG-Tyk2, and ΔStat5. Cells were also treated with the chemical dimerizer, courmermycin (CM), to activate the induced SG-Tyk2. After 24 h, cell lysates were prepared and were immunoprecipitated with anti-Tyk2 (a, b) or anti-Stat5 antibody (c, d). The immunoprecipitates were then subjected to SDS-PAGE and immunoblotted with anti-phosphotyrosine (α-PY) antibody (a, c). The filters were re-probed with anti-Tyk2 or anti-Stat5 antibody (b, d). (C) The effect of ΔStat5 on Tyk2-dependent cell proliferation. Growth of SG/dSt-5-5 cells was examined by culturing them in IL-3-free medium containing tetracycline (Tet) or IPTG in the absence (DMSO) or presence of CM. It should be noted that the parental SG-15-2 cells proliferate in IL-3-free medium containing IPTG and CM because of the CM-dependent activation of SG-Tyk2[13] (data not shown). □ Tet, △ IPTG, ○ Tet+CM, ◇ IPTG+CM.

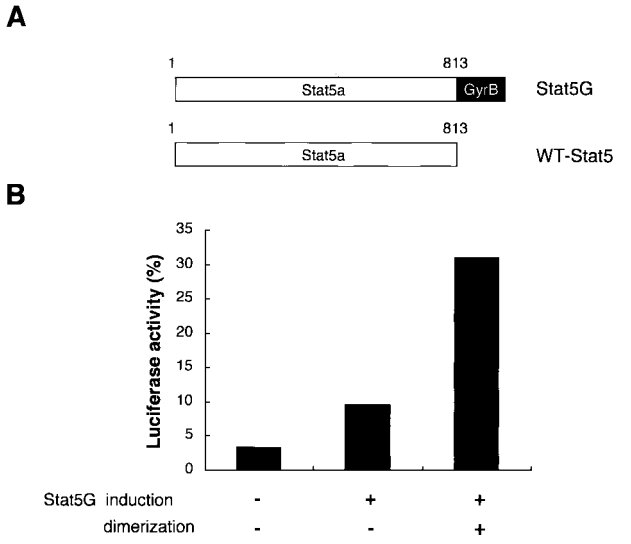

Fig. 3. Conditional activation of Stat5 by courmycin-mediated dimerization. (A) Schematic of the conditionally dimerizable Stat5a, Stat5G. Stat5G was made by adding the dimerization domain derived from the B subunit of bacterial DNA gyrase (GyrB; shaded box) at the carboxy-terminus of wild-type Stat5a (WT-Stat5). (B) *β-casein* promoter activation by Stat5G. Ba/F3-derived 6-1 cells were transiently transfected with the *β-casein* promoter-luciferase reporter plasmid and an inducible expression plasmid for Stat5G (pOPTET-puro-Stat5G). Cells were cultured in IL-3-free medium for 30 h. Stat5G expression was suppressed by tetracycline (induction −) and was induced by IPTG (induction +). Stat5G was conditionally dimerized by adding courmermycin to the culture. Data were normalized for % induction of those stimulated by IL-3 for the last 6 h. Data shown are representative of three independent experiments.

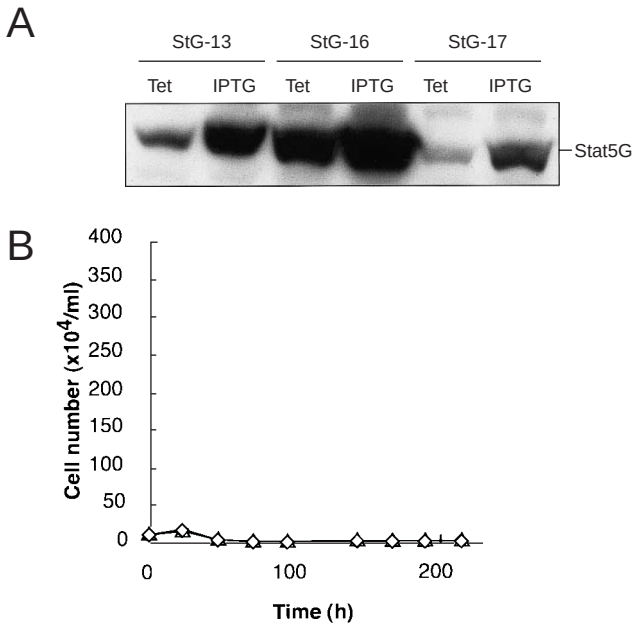

Fig. 4. Ectopic expression of a conditionally dimerizable Stat5, Stat5G, in Ba/F3 cells. (A) Ectopic expression of Stat5G in Ba/F3-derived stable transfectants. Total cell lysates prepared from the transfectants cultured in the presence of either tetracycline (Tet) or IPTG were subjected to SDS-PAGE and immunoblotted with anti-Stat5 antibody. The position of Stat5G is indicated. (B) The effect of Stat5G on Ba/F3 cell proliferation. Growth of the Stat5G-transfectant, StG-16, was examined by culturing them in IL-3-free medium containing IPTG in the absence or presence of courmermycin (CM). △ IPTG, ◇ IPTG+CM.

observations that Stat5 is another molecular component whose activation is indispensable for JAK-triggered mitogenesis.

Generation and expression of conditionally dimerizable Stat-5 in Ba/F3 cells Since STATs are known to be activated upon phosphorylation-dependent dimerization,[5, 6] we suspected that artificial dimerization of STAT with the use of a chemical dimerizer would also activate its transcriptional activity. To this end, we generated a conditionally dimerizable Stat5a in which a chemical dimerization domain derived from bacterial gyrase B (GyrB) was connected to the carboxy-terminal end of Stat5a (Fig. 3A). The cDNA encoding the Stat5a-GyrB chimera (designated here as Stat5G) was inserted into the pOPTET-puro vector. The resulting plasmid, pOPTET-puro-Stat5G, was then transiently introduced into 6-1 cells. The luciferase reporter assay using *β-casein* promoter, whose activity is regulated by Stat5, revealed that Stat5G is inducibly expressed in cells by IPTG and its transcriptional activity is potently stimulated by the chemical dimerizer, courmermycin (Fig. 3B). We concluded from the observation that Stat5G is a conditionally active Stat5, which is activated by the courmycin-mediated dimerization.

The inducible pOPTET-puro-Stat5G plasmid was next introduced into 6-1 cells. Despite extensive screening, we were not able to establish transformants in which the expression of Stat5G is strictly under the control of Tet/IPTG (Fig. 4A). However, because the activation of the expressed Stat5G is totally dependent on the chemical dimerizer, we could address the effect of conditional activation of Stat5 on Ba/F3 cell proliferation by employing these transfectants. As Fig. 4B shows (representative data obtained with the StG-16 cells), the Stat5G transfectants were incapable of proliferating in the absence of IL-3, despite forced activation of Stat5 through courmycin-mediated dimerization.

The collaboration of Ras and Stat5 in Ba/F3 proliferation The activation of either Ras or STAT alone was insufficient to induce Ba/F3 cell proliferation. We hence addressed the possible collaboration of the two molecules in triggering Ba/F3 cell proliferation in the absence of IL-

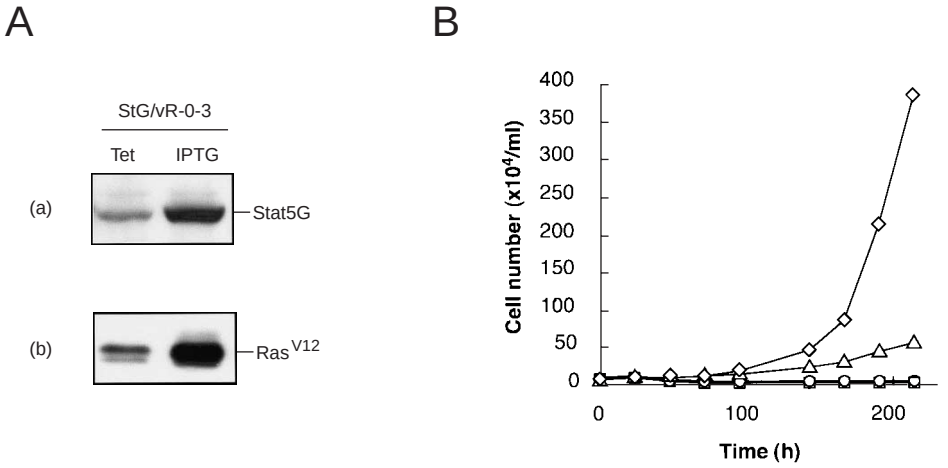

Fig. 5. Concomitant expression and activation of Ras and STAT in Ba/F3 cells. (A) Inducible expression of Stat5G and Ras^{V12} in a Ba/F3-derived stable transfectant clone, StG/vR-0-3. Total cell lysates prepared from the transfectant cultured in the presence of either tetracycline (Tet) or IPTG were subjected to SDS-PAGE and immunoblotted with anti-Stat5 antibody (a) or anti-Ras antibody (b). The positions of Stat5G and Ras^{V12} are indicated. (B) The effect of Ras^{V12} and Stat5G on Ba/F3 cell proliferation. Growth of the StG/vR-0-3 cells was examined by culturing them in IL-3-free medium containing Tet or IPTG in the absence or presence of courmermycin (CM). □ Tet, △ IPTG, ○ Tet+CM, ◇ IPTG+CM.

3. To do so, an inducible expression vector for Stat5G (pOPTET-puro-Stat5G) was introduced into the Ras^{V12} transfectant, Rv-6-10 (see Fig. 1), and two independent clones, StG/vR-0-2 and StG/vR-0-3, were established. As we expected, these double stable transfectants inducibly expressed Stat5G and Ras^{V12} following IPTG treatment (Fig. 5A). Using one of the transfectants, StG/vR-0-3, we examined the cooperative effect of Stat5 and Ras on cell growth. As demonstrated in Fig. 5B, the inducible expression of Stat5G and Ras^{V12} gave rise to very weak cell proliferation, most probably due to the activated Ras^{V12}, as shown in Fig. 1. In contrast, the activation of Stat5G by chemical dimerization with CM in the presence of Ras^{V12} induced a strong Ba/F3 cell proliferation comparable to that induced by conditional Tyk2 activation or IL-3 treatment. Again, the presence of a latent time for growth indicates that certain cellular conditioning may be required to initiate cell cycle progression in cells with activated Stat5 and Ras. Essentially the same observation was obtained when another clone, StG/vR-0-2, was examined (data not shown). Our observations thus indicate that concomitant activation of Ras and STAT suffices for cell proliferation.

DISCUSSION

Cytokine-receptor interaction activates multiple cytoplasmic signal transducers in addition to JAKs. The establishment of cells whose proliferation is strictly dependent on the activated JAK provided us with a unique opportunity to study downstream elements of JAKs in mitogenic signaling in the absence of concomitant activation of other signaling pathways that lie downstream of the cytokine receptor.

We have previously shown that JAK, when appropriately activated, is capable of generating sufficient input to promote cell proliferation.[13] This JAK-triggered mitogenesis is blocked by inhibiting either endogenous Ras or STAT activity. However, the single activation of Ras or STAT in Ba/F3 cells is insufficient to induce effective cell proliferation, although constitutively active Ras is capable of preventing apoptosis under cytokine-depleted conditions. In striking contrast, concomitant activation of Ras and STAT in Ba/F3 cells suffices for cytokine-independent proliferation. From these observations, we conclude that Ras and STAT are essential and sufficient downstream components of JAK-triggered mitogenesis. Since JAKs are rapidly activated in response to cytokine-receptor interaction, our results further suggest that the cytokine-induced mitogenic signal bifurcates into Ras and STAT pathways after activation of JAK.

We have shown that p130, a retinoblastoma tumor suppressor protein (pRB) homologue, specifically and actively inhibits cell cycle progression of hematopoietic cells, including Ba/F3.[16, 22] This in turn indicates that p130 must be inactivated when Ba/F3 cells commit to cell cycle progression in response to a mitogenic cytokine. Like pRB, the growth-inhibitory activity of p130 is considered to be regulated by phosphorylation, most likely through G1 cyclin-cyclin dependent kinases (CDKs).[23, 24] Considering these findings together with our findings

points to the notion that the Ras and STAT pathways eventually culminate in the regulation of the pRB family of tumor suppressor proteins through G1 cyclin-CDK.

Although the molecular mechanisms linking JAK and G1 cyclin-CDK remain obscure, it should be noted that D-type cyclin was originally identified as a molecule that is induced upon cytokine stimulation in a macrophage cell line.[25] More recently, Ras was reported to be involved in the induction of cyclin D.[26–28] Hence, through Ras activation, JAK may promote cyclin D-CDK activation.

The observation that the constitutively active Ras^{V12} is insufficient for cytokine-independent growth implies that effective phosphorylation/inactivation of p130 requires distinct G1 cyclin-CDK(s) in addition to cyclin D-CDK. Work with pRB[29–32] promotes cyclin E-CDK as an attractive candidate for such a kinase. In contrast to cyclin D, however, regulation of cyclin E expression is poorly understood, although it appears to involve both transcriptional and post-transcriptional mechanisms.[33–35] Our present work raises the intriguing possibility that STATs may play a role in regulating cyclin E expression. Alternatively, they may control the activities of CDK inhibitors such as $p21^{Cip1}$ and $p27^{Kip1}$, both of which neutralize the kinase activity of cyclin E-CDK through physical complex formation.[36]

The conditional, JAK-dependent or Ras/STAT-dependent cells established here should provide a powerful tool for identifying the molecular components that link the Ras and STAT pathways with cell cycle regulators.

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