Ras Is Not Required for the Interleukin 3-induced Proliferation of a Mouse Pro-B Cell Line, BaF3*

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It has been demonstrated that Ras is involved in interleukin 3 (IL-3)-stimulated signal transduction in various hematopoietic cultured cells (Satoh, T., Nakafuku, M., Miyajima, A., and Kaziro, Y. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 3314–3318; Duronio, V., Welham, M. J., Abraham, S., Dryden, P., and Schrader, J. W. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 1587–1591). However, it has not been fully understood which of IL-3-promoted cellular responses, i.e., proliferation, survival, and differentiation, requires Ras function. We employed a system of inducible expression of the dominant-negative (S17N) or dominant-active (G12V) mutant of Ras in BaF3 mouse pro-B cell line to analyze the role of Ras in IL-3-stimulated signal transduction. Induction of the dominant-negative Ras(S17N) effectively inhibited the IL-3-induced activation of c-Raf-1 and mitogen-activated protein kinase (MAPK). Furthermore, the activation of gene promoter following IL-3 stimulation was almost completely abolished when Ras(S17N) was induced. Under these conditions, Ras(S17N) exhibited no inhibitory effect on IL-3-dependent proliferation assessed by the increase of cell numbers and a mitochondrial enzyme activity. The results indicate that Ras-dependent pathways, including the Raf/MAPK/Fos pathway, are dispensable for IL-3-induced growth stimulation. When BaF3 cells were treated with a tyrosine kinase inhibitor, herbimycin A, IL-3-dependent proliferation of the cells was impaired, suggesting that tyrosine kinase-mediated pathways are critical for growth promotion. On the other hand, apoptotic cell death caused by deprivation of IL-3 was prevented by the induction of the activated mutant Ras(G12V), although the rate of cell number increase was markedly reduced. Thus, it is likely that Ras-independent pathways play important roles to facilitate the proliferation although they may not be essential for IL-3-stimulated antiapoptotic signal transduction.

In various types of cells, Ras functions as a molecular switch that regulates intracellular signaling pathways for proliferation, differentiation, and other physiological responses. Tyrosine kinase receptors, such as epidermal growth factor receptor and platelet-derived growth factor receptor, when stimulated by their specific ligands, form a complex with a variety of signal-transducing molecules including phosphatidylinositol 3-kinase, Ras-GTPase activating protein (RasGAP), 3-phospholipase C-γ1, and adaptor proteins (e.g., Grb-2, Nck, and Shc) through the interaction between specific phosphotyrosines on the receptor and Ssrc homology 2 (SH2) domains of the binding molecules (Fanti et al., 1993; Schlessinger, 1993). Among the above components of the signal-transducing complex, Shc and Grb-2 are well characterized as elements that link the receptor and a Ras-guanine nucleotide exchange factors (Ras-GEFs), such as mSos-1. Adaptors and Ras-GEFs are known to participate also in Ras regulation through non-tyrosine kinase-type receptors including interleukin 2 (IL-2), IL-3, and T cell antigen receptors (Burns et al., 1993; Cutler et al., 1993; Ravichandran et al., 1993; Sato et al., 1993; Buday et al., 1994; Reif et al., 1994; Ravichandran and Burakoff, 1994; Welham et al., 1994).

The active GTP-bound form of Ras specifically binds Ras-GAPs (Boguski and McCormick, 1993), c-Raf-1 (Avruch et al., 1994), B-Raf (Moodie et al., 1994; Vaillancourt et al., 1994), phosphatidylinositol 3-kinase (Rodriguez-Viciana et al., 1994), Ras-guanine nucleotide dissociation stimulator (Hofer et al., 1994; Kikuchi et al., 1994; Spaargaren and Bischoff, 1994), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) kinase (MEKK) (Lange-Carter and Johnson, 1994), and Rin1 (Han and Colicelli, 1995), among which Raf proteins are best characterized as direct targets of Ras (Daum et al., 1994; Marshall, 1995). After the binding of Ras, the serine/threonine kinase activity of Raf is enhanced by interaction with membrane components (Leevers et al., 1994; Stokoe et al., 1994; Dent et al., 1995; Marais et al., 1995), and then the activated Raf stimulates MEK and MAPK.

In addition to the Raf/MAPK pathway, it has been proposed that Rac-mediated pathways function downstream of Ras and are essential for the induction of transformed phenotypes by oncogenic Ras in Rat1 and NIH3T3 cells (Qiu et al., 1995). Furthermore, sequential activation of Raf and Rho upon cell stimulation to trigger the assembly of focal complexes has been reported (Nobes and Hall, 1995), suggesting a cascade of small GTPases.

In fibroblast cells, Ras is implicated in the signal transduction of normal growth as well as malignant transformation. On the other hand, in PC12 pheochromocytoma and 3T3-L1 cells, Ras stimulates the differentiation to neuronal cells and adipocytes, respectively. It has also been revealed that Ras plays a crucial role in T cell activation signaling. Furthermore, in factor-dependent hematopoietic cell lines, accumulation of the

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‡The abbreviations used are: GAP, GTPase-activating protein; CAT, chloramphenical acetyltransferase; ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated kinase; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; GEF, GDP/GTP exchange factor; GST, glutathione S-transferase; IL-2/3, interleukin 2/3; IPTG, isopropyl-β-D-thiogalactoside; jAK, Janus kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MEK, MAPK kinase; MEK kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide; RSV, Rous sarcoma virus; PAGE, polyacrylamide gel electrophoresis; SH2, Src homology 2; STAT, signal transducers and activators of transcription.
active GTP-bound Ras was detected in response to the stimulation with various cytokines including IL-2, IL-3, granulocyte/macrophage colony-stimulating factor (GM-CSF), and Steel factor, which induce both proliferation and differentiation (see Satoh et al., 1992b; Boguski and McCormick, 1993), and Lowy and Williams (1993) for reviews).

IL-3 is indispensable for survival and proliferation of a mouse pro-B cell line, BaF3. Upon binding of IL-3, the IL-3 receptor, consisting of a heterodimer of α and β subunits, triggers multiple signals, for instance, Ras-dependent, J anus kinase (J AK) signal transducers and activators of transcription (STAT) protein-mediated, and Myc-related pathways. It is likely that these signaling pathways cooperatively regulate cellular proliferation, survival, and differentiation although the role and relationship of each pathway remain unclear (see Darnell et al., 1994, Ihe et al., 1994, and Sato and Miyajima (1994) for reviews).

In this study, we investigated the roles of Ras-dependent signal transduction pathways in IL-3-induced cell growth of BaF3 cells by utilizing a system for inducible expression of dominant-negative (S17N) as well as dominant-active (G12V) mutants of Ras protein. The dominant-negative Ras(S17N) almost completely blocked the signal transduction downstream of Ras including the activation of c-Raf-1 protein and subsequent hyperphosphorylation of MAPK. Under these conditions, the cell growth was not interfered with at all, indicating that the Ras pathway may be dispensable for IL-3-promoted proliferation. It was also found that a constitutively active Ras(G12V) by itself was able to prevent the cells from apoptotic cell death caused by IL-3 deprivation.

**EXPERIMENTAL PROCEDURES**

**Materials—**Anti-Ras antiserum Has6 (Tanaka et al., 1985) is a generous gift of Takeo Tanaka (Kure National Hospital, Kure). Antibodies against MAPK (05–157), phosphotyrosine (05–321), and Shc (06–203) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-Grb-2 (MS–20–3) and anti-c-Raf-1 (sc–227) antibodies were obtained from MBL (Nagoya) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. E. coli expression plasmids for His-MAPKK and glutathione S-transferase (GST)-MAPK (K57D) (Kosako et al., 1993; Gotoh et al., 1994) were kindly provided by Eisuke Nishida (Kyoto University, Kyoto). c-fos luciferase (Fukumoto et al., 1990) was kindly provided by Kozo Kaibuchi (Nara Advanced Institute of Science and Technology, Ikoma). pCMV5-Ras(S17N) was constructed by inserting ras(S17N) cDNA (Szeberényi et al., 1990, kindly provided by Geoffrey Cooper (Dana-Farber Cancer Institute, Boston, MA)) into pCMV5 (Andersson et al., 1989). LacSwitch inducible mammalian expression system (217450), including expression plasmids pOPRSVICT and p35S, was purchased from Stratagene. Mouse IL-3 is a generous gift of Atsushi Miyajima and Satish Menon (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA). Herbimycin A is a generous gift of Yoshimasa Uehara (National Institutes of Health, Bethesda, MD).

**Cell Culture—**BaF3, N6, and V2 cells were cultured in RPMI 1640 supplemented with 10% (v/v) fetal calf serum and mouse IL-3. For N6 and V2 cells, G418 (1 mg/ml) and hygromycin (1 mg/ml) were included in the culture medium.

Isolation of BaF3 Transfectants—DNA encoding a dominant-negative (S17N) (Szeberényi et al., 1990) or dominant-active (G12V) (Satoh et al., 1992) mutant of human c-Ha-ras was inserted into NotI cloning sites instead of chloramphenicol acetyltransferase (CAT) gene within a mammalian expression vector pOPRSVICT. The ras expression plasmids were introduced into BaF3 cells with p35S, which encode a constitutive expression, by electroporation, and appropriate transfected clones were isolated using G418 (1.5 mg/ml) and hygromycin (1 mg/ml) essentially as described elsewhere (Satoh et al., 1993). Clones termed N6 (for Ras(S17N)) and V2 (for Ras(G12V)) were used for further experiments.

Ras proteins were induced by adding 5 mM IPTG into the culture medium.

**Analysis of Ras-bound Guanine Nucleotide—**Analyses of Ras-bound guanine nucleotide in N6 cells were performed as described elsewhere (Satoh et al., 1993).

Immunoprecipitation and Immunoblotting—Cells were dissolved in IP buffer (50 mM Hepes-NaOH (pH 7.3), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 2 mM MgCl2, 1 mM EDTA, 100 mM NaF, 10 mM Na3P04, 20 mM β-glycerophosphate, 1 mM Na2VO3, 1 mM phenylmethlysulfonfluryl fluoride, 10 μM aprotinin, 1 μM leupentin, and the precipitate was washed twice with buffer (50 mM Tris-HCl (pH 7.5), 20 mM NaCl, 150 mM NaCl), and then once with kinase buffer (20 mM Hepes-NaOH (pH 7.0), 0.1 M MgCl2, 1.5 mM Mg2+, 15 μg/ml aprotinin, 0.15 mM Na2VO3, 33.5 μM His-MAPKK, 100 μM GST-MAPK(K57D), 0.2 μM [γ-32P]ATP (37 TBq/mol) for 20 min at 30°C. The proteins were analyzed by SDS-PAGE and autoradiography.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrasodium Bromide (MTT) Assay—Cells were lysed in LOM (20 mM Hepes-NaOH (pH 7.3), 10 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 25 mM Na3P04, 20 mM β-glycerophosphate, 10 μM leupentin, 10 μM pepstatin A), and the precipitate was washed twice with wash buffer (50 mM Tris-HCl (pH 7.5), 20 mM NaCl, 150 mM NaCl), and then once with kinase buffer (20 mM Hepes-NaOH (pH 7.0), 0.1 M MgCl2). The precipitated proteins were subjected to MTT kinase assay within a reaction mixture containing 20 mM Hepes-NaOH (pH 7.5), 1.5 mM Mg2+, 15 μg/ml aprotinin. Proteins (0.15 mg/ml Na2VO3, 33.5 μM His-MAPKK, 100 μM GST-MAPK(K57D), 0.2 μM [γ-32P]ATP (37 TBq/mol) for 20 min at 30°C. The proteins were analyzed by SDS-PAGE and autoradiography.

RESULTS

To clarify the role of Ras in IL-3-stimulated signal transduction for cell growth, we established cell lines in which the dominant-negative (S17N) or dominant-active (G12V) form of Ras protein is expressed in an inducible manner. Mouse IL-3-dependent BaF3 pro-B cells were used as the parental cell line. The cells were transfected with two expression plasmids; one for Ras and the other for the repressor of E. coli lac operon (termed pOPRSVICT) and p35S, respectively. The repressor binds to the operator sequence that was inserted into the Rous sarcoma virus (RSV)-derived promoter of pOPRSVICT-Ras to suppress the expression. When IPTG is added to the medium, the repressor is released from the lac operator sequence, and transcription of the exogenously introduced cDNA is initiated. Two stable transfecteds designated N6 (for Ras(S17N)) and V2 (for Ras(G12V)) were selected and characterized in detail. Fig. 1 shows the expression of the mutant Ras proteins in N6 and V2 cells upon stimulation with IPTG. In both cases, expression of the mutant Ras was detected after 4 h of treatment with IPTG and reached a plateau after 16 h. Faint bands of endogenous Ras were detected above Ras(S17N) in A, and below Ras(G12V) in B. It seems likely that a band of unmodified Ras(S17N) appears at the same position as the endogenous Ras after 24-h induction. We consider that it does not interfere with the action
Evidence for the specific interaction between Ras and c-Raf-1 proteins was provided in various types of mammalian cells using several different methods, such as co-immunoprecipitation, affinity chromatography, and yeast two-hybrid systems (Koide et al., 1993; Avruch et al., 1994; Daum et al., 1994). Furthermore, it has been demonstrated that the blockade of c-Raf-1 function resulted in the failure of Ras to stimulate proliferation (Klich et al., 1991), indicating that c-Raf-1 acts downstream from Ras. Hence, we examined whether the dominant-negative Ras(S17N) sufficiently inhibited the Ras-mediated pathway by comparing the activation of c-Raf-1 protein in response to IL-3 stimulation between IPTG-treated and untreated N6 cells. Endogenous c-Raf-1 protein was immunoprecipitated with a specific antibody, and the kinase activity of the protein was assayed using recombinant MAPK kinase (MAPKK or MEK) as a substrate and by measuring the incorporation of radioactive phosphate into recombinant kinase-negative MAPK added to the reaction mixture. As illustrated in Fig. 2A, IL-3 rapidly triggered the activation of c-Raf-1 protein in N6 cells in the absence of IPTG, indicating that Ras/Raf pathway functions downstream of the IL-3 receptor. When the cells were treated with IPTG for 16 h, the induction of c-Raf-1 activation was completely diminished, suggesting that the induced Ras(S17N) interferes with the function of endogenous Ras.

Then, we investigated the phosphorylation and subsequent activation of one of the endogenous MAPKs, ERK2, following IL-3 stimulation, which can be detected by immunoblotting as the appearance of mobility-shifted bands corresponding to phosphorylated ERK2. As shown in Fig. 2B, IL-3 induced the phosphorylation of ERK2 in a similar time course as c-Raf-1 activation in N6 cells without IPTG pretreatment, whereas, in cells treated with IPTG for 16 h prior to the addition of IL-3, ERK2 was no longer activated.

Analyses of Ras-bound guanine nucleotide in N6 cells were carried out to confirm that Ras(S17N) actually inhibits the formation of an active form of Ras within the cell since it is well known that only a GTP-bound conformation can transduce the signal to downstream targets including c-Raf-1. The molar ratios of the GTP-bound form were: 2.6% in control and 18.5% in IL-3-stimulated N6 cells without IPTG pretreatment, and 1.8% in control and 5.4% in IL-3-stimulated cells after the induction of Ras(S17N) by IPTG, respectively.

We next examined the effect of Ras(S17N) on IL-3-enhanced transcription from c-fos promoter using c-fos-luciferase as a reporter plasmid. As shown in Fig. 3A, IL-3 stimulated the luciferase activity in control N6 cells, which was significantly reduced when Ras(S17N) was induced by IPTG treatment of the cell. Co-transfection of increasing amounts of a plasmid that expresses Ras(S17N) constitutively, designated pCMV5-Ras(S17N), also diminished IL-3-promoted transcription from c-fos promoter (Fig. 3B). The results indicate that c-fos induction in response to IL-3 stimulation is Ras-dependent, and the induction of Ras(S17N) with IPTG in N6 cells is sufficient for interfering with this signaling pathway.

Then, we analyzed the effects of dominant-negative Ras(S17N) on IL-3-dependent cell survival and long-term proliferation (Fig. 4). N6 cells showed IL-3-dependent characteristics: they were not able to survive and proliferate in the absence of IL-3, while, in the presence of increasing concentrations of IL-3, MTT-reducing activity was detected, and the cells continuously proliferated. Interestingly, even when the dominant-negative Ras(S17N) was induced with IPTG, the growth properties did not change at all in spite of the almost complete abrogation of IL-3-dependent stimulation in the Raf/MAPK/fos pathway as described in Figs. 2 and 3. The results indicate that the Ras pathway is not essential for IL-3-induced growth stimulation in BaF3 cells.

Various adaptor proteins and Ras-GEFs are involved in tyrosine kinase receptor-mediated activation of Ras (Schlessinger, 1993). Similarly, lymphokine receptors, for example IL-2 and IL-3 receptors, activate Ras through the interaction with tyrosine phosphorylated proteins, adaptors, and Ras-GEFs (Burns et al., 1993; Cutler et al., 1993; Sato et al., 1993; Ravichandran and Burakoff; 1994; Welham et al., 1994). To test whether Ras(S17N) or Ras(G12V) might affect IL-3 receptor-stimulated responses of Ras regulators, we compared tyrosine phosphorylation of Shc and its interaction with Grb-2 in BaF3, N6, and V2 cells. As shown in Fig. 5, when stimulated with IL-3 for 7 min, tyrosine phosphorylation of both species of Shc (p46 and p52) and Shc/Grb-2 association were observed in all of the above cell lines. A tyrosine-phosphorylated protein with a molecular weight of 150,000 that was co-immunoprecipitated with Shc (Buday et al., 1994) was also detected in these cell lines.
Even though Ras(S17N) or Ras(G12V) was induced by IPTG treatment for 16 h, these responses were unaffected, suggesting that the mutant Ras proteins do not interfere with the signal transduction pathways upstream of Ras. To clarify whether IL-3-induced activation of tyrosine kinases and their interaction with downstream signaling molecules are required for cell growth, we next analyzed the effect of a tyrosine kinase-specific inhibitor, herbimycin A. As illustrated in Fig. 6A, treatment of BaF3 cells with herbimycin A prior to the stimulation completely blocked both IL-3-induced tyrosine phosphorylation of Shc and Shc/Grb-2 association. Moreover, herbimycin A-treated BaF3 cells were unable to survive and proliferate even in the presence of IL-3 (Fig. 6B). The results suggest that tyrosine kinase-mediated pathways are critical for the induction of cell growth in response to IL-3.

Although Ras is not essential for IL-3-dependent growth stimulation, it is possible that Ras may be involved in the growth signaling pathway. To clarify this point, we next studied the effects of dominant-active Ras(G12V) using V2 cells. In V2 cells, Ras(G12V) was induced after a 4-h treatment with IPTG as shown in Fig. 1. We measured MEK kinase activity of immunoprecipitated endogenous c-Raf-1 protein and the mobility retardation of ERK2 following the induction of Ras(G12V) in IL-3-deprived V2 cells. As shown in Fig. 7, both MEK kinase activity of c-Raf-1 and the hyperphosphorylation of ERK2 were markedly enhanced by Ras(G12V) even in the absence of IL-3 stimulation. In addition, we detected increased transcription from c-fos promoter in IPTG-induced V2 cells in the absence of IL-3 using the luciferase assay (data not shown). Under these conditions, we tested whether the cells are able to survive and proliferate.

Fig. 8 illustrates the fragmentation of chromosomal DNA characteristic of apoptotic cell death. IL-3 starvation for 48 h
caused apoptosis in both N6 and V2 cells. Whereas Ras(S17N) showed no effect on the induction of DNA fragmentation, Ras(G12V) effectively prevented the cells from the apoptotic cell death. The findings imply the participation of Ras protein in antiapoptosis signaling although Ras is not essential for IL-3-dependentsurvival of the cells.

**DISCUSSION**

Although Ras is activated upon IL-3 stimulation, it has not been clear whether the function of Ras is required for IL-3-induced proliferation of hematopoietic cell lines. A dominant-negative mutant Ras(S17N), which binds to Ras-GEFs tightly to prevent its action toward endogenous Ras, has been utilized widely to evaluate whether Ras is essential for a particular signal transduction pathway (Feig and Cooper, 1988). In fibroblast cell lines, for example NIH3T3 cells, the dominant-negative Ras(S17N) efficiently blocks growth factor- or serum-induced DNA synthesis and cell cycle progression (Feig and Cooper, 1988; Cai et al., 1990). The mutant also inhibits nerve growth factor-induced neuronal differentiation of PC12 cells without affecting the cell growth (Szeberényi et al., 1990). The results indicate that the function of Ras signaling pathway may differ depending on cell types.

IL-3 triggers the activation of multiple signal transducing molecules. In the present study, we took advantage of an inducible expression system for dominant-negative and dominant-active mutant Ras proteins to assess the function of Ras in these pathways. Only Ras-independent pathways are activated when dominant-negative Ras(S17N) is induced in the presence of IL-3, whereas only Ras-dependent pathways are activated when dominant-active Ras(G12V) is induced in the absence of IL-3. The system allowed us to evaluate the function
of Ras-dependent and Ras-independent signaling pathways in IL-3-dependent pro-B cell line BaF3.

In this paper, it was demonstrated that the cells displayed normal phenotypes, that is IL-3-dependent survival and proliferation when the dominant-negative form of Ras(S17N) was expressed sufficiently to inhibit the Ras-dependent signal transduction. On the other hand, when the dominant-active form of Ras(G12V) was present, the cells were capable of escaping apoptotic cell death and proliferating without IL-3 stimulation although the growth rate was significantly lowered. Furthermore, it was shown that tyrosine kinase-specific inhibitor, herbimycin A, blocked the cell growth as well as Shc/Grb-2 interaction. Thus, in conclusion, it is likely that tyrosine kinase-mediated, but Ras-independent signaling pathways are essential for transmitting sufficient signals for the induction of cell growth, whereas the Ras-dependent pathway is dispensable.

Several signaling molecules other than Raf family proteins, for instance, phosphatidylinositol 3-kinase (Rodríguez-Viciana et al., 1994), Rac-guanine nucleotide dissociation stimulator (Hofer et al., 1994; Kikuchi et al., 1994; Spaargaren and Bischoff, 1994), and MEKK (Lange-Carter and Johnson, 1994), have been proposed to be direct targets of Ras although it is not clear whether these molecules function within BaF3 cells. Furthermore, Rac-mediated pathways, which are also downstream from Ras, were recently reported to be independent of the Raf kinase cascade and essential for malignant transformation of fibroblast cells (Qi et al., 1995). We consider that signaling pathways targeted by Ras-GTP other than the Raf/MAPK pathway must be blocked as well if the Raf/MAPK pathway is completely abolished because all effectors of Ras are thought to interact with only GTP-bound active conformation of Ras, and Ras(S17N) inhibits extracellular signal-dependent accumulation of this conformation. We actually observed that the formation of Ras-GTP within N6 cells upon IL-3 stimulation was limited to a lower level when Ras(S17N) was induced. However, if the affinities between Ras and different target molecules are different, we cannot exclude completely the possibility that the residual Ras activity may be sufficient for stimulating a downstream signaling pathway other than the Raf/MAPK pathway, which is essential for growth stimulation.

We described that the activation of the endogenous c-Raf-1 and ERK2 elicited by IL-3 treatment was reduced considerably when Ras(S17N) was induced. The results also indicate that, in BaF3 cells, the Raf/MAPK pathway is dependent on Ras, which is similar to the signal transduction of platelet-derived growth factor, insulin, and nerve growth factor (de Vries-Smits et al., 1992; Thomas et al., 1992; Wood et al., 1992), but distinct from the case of epidermal growth factor, in which multiple pathways are responsible for the activation of MAPK (Burgering et al., 1993). Likewise, the transcriptional activation of the c-fos gene is dependent on Ras function in BaF3 cells.

Okuda et al. (1994) recently reported that 32D myeloid cells failed to proliferate in response to IL-3 when the dominant-negative Ras(S17N) was induced, whereas they were able to survive for more than 2 weeks in the presence of Ras(S17N) and IL-3. In addition, they found that granulocyte colony-stimulating factor (G-CSF)-induced differentiation to neutrophils was not affected by Ras(S17N) (Okuda et al., 1994). The discrepancy between their results and ours may be due to the difference in the signal transduction networks between the two types of hematopoietic cells.

IL-3 and GM-CSF receptors share the common β subunit, which plays a pivotal role in signal transduction. From analyses using a set of deletion mutants of the common β subunit, a cytoplasmic region responsible for Ras activation was identified (Sato et al., 1993). Kinoshita et al. (1995) have recently reported that mutant GM-CSF receptors lacking the ability to stimulate the Ras/Raf pathway failed to suppress apoptosis, but they were still able to exert DNA synthesis. In addition, they showed that the activated Ras(G12V) could overcome the mutants’ inability to prevent the cell death. In our experiments, the expression of Ras(S17N) did not result in apoptosis (Fig. 8), and the cells could proliferate continuously (Fig. 4). Hence, it is possible that a Ras-independent pathway that is sufficient for the survival of the cells may be activated through the C-terminal domain of the common β subunit.

In IL-2 signal transduction, it has been proposed that three distinct signaling pathways mediated by Bcl-2, Myc, and a tyrosine kinase Lck, respectively, are regulated by IL-2 receptor (Miyazaki et al., 1995). Analyses using deletion mutants of the IL-2 receptor β subunit have revealed that the activation of a tyrosine kinase is required for the induction of Ras-GTP formation, as well as the fact that a mutant that is incapable of stimulating the Ras pathway can stimulate DNA synthesis (Satoh et al., 1992a). The results imply that Ras-dependent signals may not crucial for proliferation also in IL-2 signaling system.

Several kinds of Ras-independent pathways, for example the J AK/STAT pathway and myc-related pathway, have been reported. A member of the J AK family tyrosine kinases, J AK2, is known to bind to the membrane-proximal region of the common β subunit of IL-3 receptor. Following IL-3 stimulation, J AK2 is activated and subsequently phosphorylates STAT5, which induces the transcription of specific genes (Silvennoinen et al., 1993; Quelle et al., 1994; Azam et al., 1995; Mui et al., 1995) although the role of this pathway has not been fully manifested. Furthermore, it is possible that adaptors, such as Shc and Grb-2, which function downstream from tyrosine kinases, may also regulate a Ras-independent signaling cascade for cell growth. Clarification of the role of Ras in the network of multiple signaling pathways, including proliferation and differentiation, stimulated through lymphokine receptors must await further investigation.

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