

Downstream effectors of oncogenic ras in multiple myeloma cells

Liping Hu, Yijiang Shi, Jung-hsin Hsu, Joseph Gera, Brian Van Ness, and Alan Lichtenstein

Ectopic expression of mutated K-ras or N-ras in the interleukin 6 (IL-6)-dependent ANBL6 multiple myeloma cell line induces cytokine-independent growth. To investigate the signaling pathways activated by oncogenic ras that may stimulate IL-6-independent growth, we compared ANBL6 cells stably transfected with mutated K or N-ras genes with wild-type ras-expressing control cells identically transfected with an empty vector. Upon depletion of IL-6, both mutated ras-containing myeloma lines demonstrated constitutive activation of mitogen-activated extracellular kinase 2 (MEK)/extracellular signal-regulated kinase (ERK), phosphatidylinositol-3 kinase (PI3-ki-

nase)/AKT, mammalian target of rapamycin (mTOR)/p70S6-kinase, and nuclear factor kappaB (NF-kB) pathways. In contrast, signal transducer and activator of transcription-3 (STAT-3) was not constitutively tyrosine phosphorylated in mutant ras-expressing cells. We used several maneuvers in attempts to selectively target these constitutively active pathways. The mTOR inhibitors rapamycin and CCI-779, the PI3-kinase inhibitor LY294002, and the MEK inhibitor PD98059 all significantly curtailed growth of mutant ras-containing cells. Farnesyl transferase inhibitors, used to target ras itself, had modest effects only against mutant N-ras-containing cells. Growth of mutant N-ras-

containing myeloma cells was also inhibited by acute expression of the *IKB* superrepressor gene, which abrogated NF-kB activation. These results indicate that several pathways contributing to stimulation of cytokine-independent growth are activated downstream of oncogenic ras in myeloma cells. They also suggest that therapeutic strategies that target these pathways may be particularly efficacious in patients whose myeloma clones contain ras mutations. (Blood. 2003;101:3126-3135)

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Introduction

Activating mutations in N- and K-ras occur in multiple myeloma (MM) with a frequency approaching 50%.¹⁻⁵ The specific association of these mutations with Durie-Salmon stage III disease and plasma cell leukemia,^{1,6} as well as with disease progression,⁵ suggest they impart an aggressive phenotype to their myeloma clones. Gene transfer studies support this notion as transfection of interleukin 6 (IL-6)-dependent myeloma cells with oncogenic N- or K-ras induces proliferative and antiapoptotic signals that allow for IL-6-independent growth.^{7,8}

Ras is a signaling protein that switches from the inactive guanosine 5'-diphosphate (GDP)-bound state to the active guanosine 5'-triphosphate (GTP)-bound state after growth factor stimulation (reviewed in Rebollo and Martinez⁹). The point mutations of N-ras or K-ras identified in myeloma result in a constant GTP-bound state and constitutive activity. In selected transfection models, oncogenic ras has the potential for activating one or more downstream signaling cascades, such as mitogen-activated extracellular kinase (MEK)/extracellular signal-regulated kinase (ERK),¹⁰ phosphatidylinositol-3 kinase (PI3-kinase)/AKT,¹¹ mammalian target of rapamycin (mTOR)/p70S6 kinase,¹² and nuclear factor-kappaB (NF-kappaB).¹³ Since some of these cascades have also been previously shown to promote growth of myeloma cells, they may explain the ability of mutated ras to induce an aggressive

proliferative phenotype in myeloma cells. Since these substrate pathways of oncogenic ras have not yet been elucidated in myeloma cells, we initiated the current study to identify the pathways, exploiting the previously described ANBL6 myeloma cell line stably transfected with mutant N- or K-ras genes.

An additional rationale for our study was the recent development of targeted molecular therapy that is capable of attacking specific signal transduction pathways. Since oncogenic ras and its downstream constitutively activated pathways are likely to confer a proliferative advantage, they are especially attractive targets for therapy in myeloma. The results of our study indicate ras-dependent activation of the MEK/ERK, PI3-kinase/AKT, mTOR/p70S6-kinase, and NF-kappaB pathways. In addition, several inhibitors that target these pathways induced significant cytoreductive effects in mutated ras-containing myeloma cells.

Materials and methods

Cell lines and experimental design

The ANBL-6 parent and its transfected lines have been previously described in several publications.^{7,8,14} Briefly, ANBL-6 was stably transfected by retroviral transfection with virus expressing N-ras or K-ras genes

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mutated at codon 12 as described.⁸ ANBL-6 cells were also identically transfected with retrovirus lacking the transgene to serve as a control. G418-resistant populations arose after approximately 1 month of in vitro selection. To avoid artifacts of clonal selection, all studies were performed on mixed polyclonal cell populations. Expression of retroviral ras transcripts was confirmed by Northern analysis.⁸ The 3 ANBL-6 cell lines (wild-type-, mutant N-ras-, and mutant K-ras-expressing) were maintained in culture with continuous exposure to IL-6 added at 100 U/mL. While cultured in IL-6, the 3 lines demonstrated comparable growth rates and viabilities. To test constitutive signaling, our experimental design used IL-6 depletion. At time 0, IL-6 was depleted from all 3 lines, and signaling was studied 24, 48, or 72 hours later. During this time frame, viabilities of all 3 lines were comparable (see "Results").

Reagents

Recombinant IL-6 was purchased from R&D Systems (Minneapolis, MN). Phosphospecific antibodies were obtained from New England Biolabs (Beverly, MA). Rapamycin and CCI-779 were kind gifts from Wyeth-Ayerst (Pearl River, NY). They were diluted in 100% ethanol. Farnesyl transferase inhibitors FTI-277 and FPT III were purchased from Calbiochem (San Diego, CA). All other reagents were purchased from Sigma (St Louis, MO) unless otherwise described. In all experiments using inhibitors, final concentrations of all solvents were maintained at lower than 0.1%.

Western blot analysis

Protein was extracted and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described.¹⁵ Proteins were transferred to polyvinylidene difluoride membranes and phosphorylated proteins were detected using phospho-specific antibodies; in addition, total proteins were detected as previously described.¹⁵ Relative expression was determined by densitometry.

AKT kinase assay

The in vitro kinase assay used a nonradioactive kit purchased from New England Biolabs. AKT was first immunoprecipitated from cell extracts and then incubated with glycogen synthase kinase-3 (GSK-3) fusion protein in the presence of adenosine triphosphate (ATP) and kinase buffer. AKT-dependent GSK-3 phosphorylation was then measured by immunoblotting with a phosphoGSK-3 antibody that recognizes GSK-3 when phosphorylated.

ERK kinase assay

For this in vitro kinase assay, we used a kit purchased from Cell Signaling Technology (Beverly, MA). Briefly, active p44 and p42 ERK kinase were first immunoprecipitated from cell lysates with a phospho-specific antibody. The immunoprecipitates were then incubated with Ets-like transcription factor-1 (ELK-1)-glutathione S-transferase (GST) fusion protein. ERK-dependent ELK-1 phosphorylation was then measured by immunoblotting with a phospho-ELK-1 antibody that recognizes ELK-1 when phosphorylated on serine 383.

Flow cytometry for cell cycle analysis and apoptosis

For cell cycle analysis, myeloma cells were stained with hypotonic propidium iodide (50 μ g/mL in 0.1% sodium citrate) and 0.1% Triton X-100 for 1 hour at 4°C. Cells were kept in the dark at 4°C before analysis. Cell cycle distribution was determined by analyzing 10 000 to 15 000 events on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The DNA data were fitted to a cell cycle distribution analysis by use of the MODFIT program (Verity Software House, Los Angeles, CA) for MAC V2.0 (Macintosh, Seattle, WA). The percentage of cells in apoptosis was determined by enumeration of a sub-G₁ peak or by neoexpression of membrane annexin-V. A positive control for detection of apoptosis used an 18-hour incubation with anti-fas antibody (clone CH11; Upstate, Charlottesville, VA).

MTT assay

The methyl thiazolyl tetrazolium (MTT) assay was performed as described previously.¹⁵ Briefly, 1 to 2 $\times 10^4$ IL-6-depleted targets in 0.1 mL complete medium were dispensed into a 96-well microtiter plate. Drugs were added to appropriate concentrations in 100 μ L. After 48 hours of culture, 100 μ L medium was removed, and 20 μ L MTT (5 mg/mL stock) was added to each well. The plates were incubated overnight to solubilize the formazan dye. The absorbance ($A_{570\text{ nm}}$) of each well was measured the next day with a microplate enzyme-linked immunosorbent assay (ELISA) reader equipped with a 570-nm filter. Quadruplicate wells were run for each group, and the standard deviation (SD) of each group was always less than 5% of the mean. The dose of drug inducing 50% inhibition from control (ID_{50}) was determined from extrapolation of the results of the MTT assay, whereby the percentage of control was plotted versus concentration of drug.

Transfection with IKB-sr

An adenoviral vector that expresses an inhibitor of kappaB superrepressor (Ad-IKB-sr), in which serines 32 and 36 of IKB- α have been mutated to alanines, thus preventing phosphorylation and dissociation from NF-kappaB (NF- κ B) and its subsequent degradation,¹⁶ as well as the control vector (Ad-cytomegalovirus [Ad-CMV]), were amplified and titered in 293 cells. Transgene expression in the Ad-IKB-sr vector is driven by the CMV early/intermediate promoter/enhancer. The control vector, Ad-CMV, is identical to Ad-IKB-sr, but lacks a transgene insert. All adenoviral vectors were expanded and purified from 293 cells and subsequently titered. Adenoviral infection of myeloma cells was accomplished as previously described.¹⁷ Briefly, mutant N-ras-containing cells were transduced with adenovirus at varying multiplicities of infection (MOIs) for 2 hours. Adenovirus was then washed away, and cells were resuspended in media with low fetal calf serum (FCS) overnight to minimize proliferation. Cells were studied by MTT assay or flow cytometry at 24 and 48 hours after infection.

KappaB reporter gene assays

A kappaB-responsive plasmid (p5x-kB-luc) in which 5 copies of the kB-response element drives expression of firefly luciferase was purchased from Invitrogen (Carlsbad, CA). An identical plasmid that only lacked the 5 copies of the kappaB-response element was used as a control. The pRL-SV40 plasmid, in which *Renilla* luciferase is constitutively expressed under the regulation of the simian virus-40 (SV40) promoter/enhancer was purchased from Promega (Madison, WI). Myeloma cells were transfected with the p5x-kB-luc, control plasmid (lacking the kB elements), or pRL-SV40 plasmid by use of Lipofectamine Plus (Life Technologies, Gaithersburg, MD) in serum-free media according to the manufacturer's instructions. Protein was extracted 24 and 48 hours after transfection, and firefly and *Renilla* luciferase were measured by means of a Dual Luciferase Assay kit (Promega), according to the manufacturer's instructions, on the TD20/20 tube luminometer (Turner Designs, Sunnyvale, CA). Firefly luciferase activity was normalized to *Renilla* luciferase expression.

Electrophoretic mobility shift assays (EMSAs)

Wild-type and mutant kappaB oligonucleotide probes were purchased from Santa Cruz Biotechnology (CA). We combined 15 μ g nuclear protein with end-labeled, double-stranded kappaB oligonucleotide probe, 1 μ g poly-dIdC (Amersham Biotech, Piscataway, NJ), 1 μ g bovine saline albumin (BSA), and 5 mM spermidine in a final reaction volume of 20 μ L for 20 minutes at room temperature. The DNA-protein complex was run on a 4% nondenaturing polyacrylamide gel with 0.4 \times Tris (tris(hydroxymethyl)aminomethane) borate EDTA (ethylenediaminetetraacetic acid) (TBE) running buffer prior to subsequent autoradiography. Cold competition experiments were performed with a 100-fold molar excess of cold wild-type or cold mutant kappaB oligonucleotides. For supershift assays, nuclear protein was preincubated with specific or control antibodies (6 μ g) for 1 hour at room temperature.

Statistics

The *t* test was used to determine significance of differences between groups. Western blot experiments that compared expression of phosphorylated signal proteins were performed at least 3 separate times. For each experiment, densitometric analysis determined relative amounts of expression, with expression in wild-type ras control cells arbitrarily set at 1. The mean (\pm SD) relative levels of expression were used to determine significance of differences.

Results

Mutant ras expression relieves the IL-6 requirement for growth of ANBL6 myeloma cells

To investigate the differential signal cascades activated by oncogenic ras in myeloma cells, we studied the ANBL6 myeloma cell line that was stably transfected with the same constitutively active mutated ras genes found in MM patients, oncogenic K-ras or N-ras mutated at codon 12.¹⁴ The parental ANBL6 cell line, expressing endogenous wild-type ras, was identically transfected with an empty vector to serve as the control. The ANBL6 parental line is reported to be IL-6 dependent and ceases growth when depleted of exogenous IL-6.¹⁸ However, the mutated ras-expressing lines are reported to be IL-6 independent.^{7,8} To confirm these previously reported characteristics of cell growth, we depleted IL-6 from the 3 MM cell lines and studied them over the ensuing 12 days. Their growth was comparable in the presence of 100 U/mL recombinant IL-6 and during the first 3 days of IL-6 depletion. However, as shown in Figure 1, significant ($P < .05$) differences in viable cell recovery were detected by 4 days of IL-6 depletion. Thereafter, the empty vector-transfected control cells demonstrated continued inhibition of growth while both N-ras- and K-ras-mutated cells maintained progressive IL-6-independent growth. By 10 to 12 days, few viable wild-type-expressing cells were identified, while both oncogenic K-ras- and N-ras-expressing myeloma cells demonstrated doubling times of approximately 3 to 4 days. These results confirm previous reports^{7,8} that expression of oncogenic ras induces IL-6-independent growth in MM cells. The progressive growth of both mutant ras-containing cell lines continued unabated in the presence of anti-IL-6 and anti-IL-6 receptor antibodies (not shown), ruling out the possibility that mutated N-ras or K-ras induces proliferative signals via autocrine IL-6 stimulation.

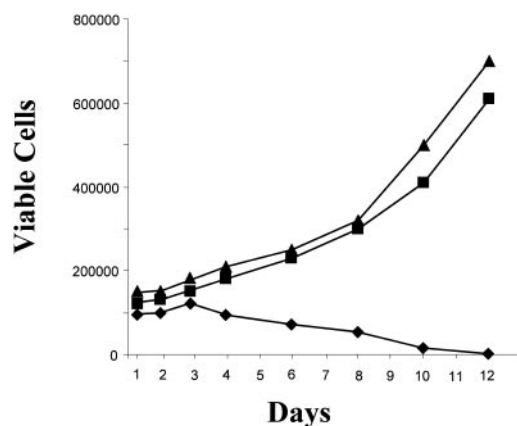


Figure 1. Effect of oncogenic ras expression on IL-6-independent growth of myeloma cells. ANBL6 cells stably transfected with empty vector (◆), mutated N-ras (▲), or mutated K-ras (■) were cultured in the absence of IL-6, and viable cell counts were performed over the ensuing 12 days. Data are means of 3 separate experiments; SDs were always less than 5% of the means.

Identification of downstream signal cascades activated by oncogenic ras in MM cells

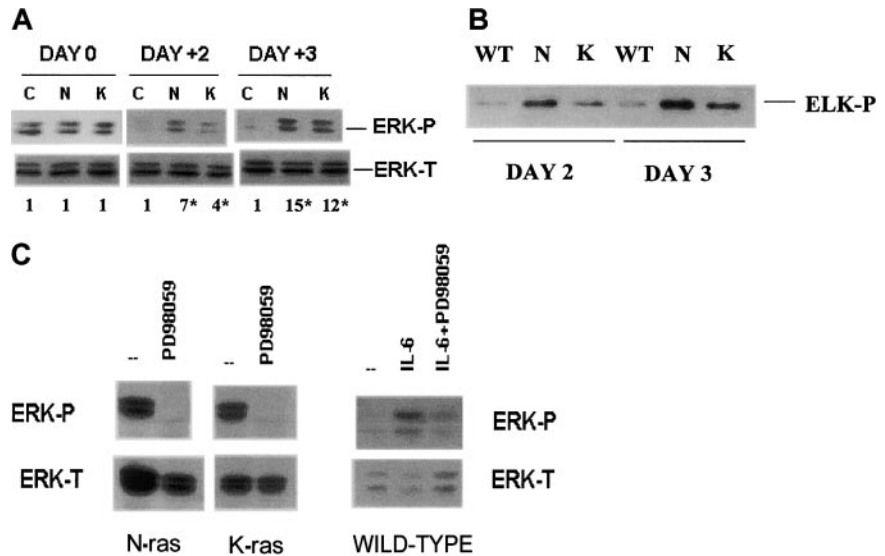
Ras signaling through ERK. Because the predominant effect of mutant ras expression was to allow continued growth in the absence of IL-6, we reasoned that the growth-promoting ras-activated signal pathways would be maintained in oncogene-expressing cells but would not be present in wild-type cells upon IL-6 depletion. Accordingly, the 3 cell lines were depleted of IL-6 and studied repeatedly over 72 hours. Within this time frame, there were no significant differences in cell recovery (Figure 1). Furthermore, the viability of wild-type-expressing cells ($93\% \pm 3\%$ at 48 hours and $88\% \pm 4\%$ at 72 hours; means \pm SDs of 5 separate experiments) was comparable to oncogenic ras-transfected cells ($96\% \pm 3\%$ and $91\% \pm 3\%$, respectively, for mutated N-ras; and $95\% \pm 2\%$ and $90\% \pm 3\%$ for mutated K-ras-containing cells) as determined by trypan blue analysis.

Phosphorylation and activation of ERK/mitogen-activated protein (MAP) kinases occur downstream of ras in IL-6-stimulated MM cells^{19,20} and are critical for their IL-6-dependent growth.²⁰ We, thus, first examined ERK phosphorylation in the ANBL6 MM model. Figure 2A demonstrates a representative experiment in which, at time 0 (day 0), following continuous culture in 100 U/mL IL-6, phosphorylation of p42 and p44 ERK is comparable among wild type-expressing control (C) cells and oncogenic N-ras (N)- and K-ras (K)-expressing ANBL6 MM cells. Following IL-6 depletion for 48 hours (day +2) and 72 hours (day +3), there was no alteration in expression of total ERK in any of the 3 cell lines. However, both p42 and p44 ERK kinases became dephosphorylated in control cells at both time points, while phosphorylation was maintained in both mutant ras-containing cell lines. The experiment shown in Figure 2A was performed 3 separate times. By densitometric analysis, the mean expressions of phosphorylated ERK in these 3 experiments are shown at the bottom of Figure 2A, with control wild-type cells set arbitrarily at 1. The differences between ERK phosphorylation in wild-type control cells and both N-ras and K-ras cells were significant ($P < .05$) at both day +2 and day +3.

Further support for these differences in ERK activity was obtained from results of an ERK in vitro kinase assay. We immunoprecipitated activated ERK from cells after 48 or 72 hours of IL-6 depletion and tested its ability to phosphorylate the ERK substrate ELK-1. As shown in Figure 2B, mutated N-ras and K-ras cells demonstrated significantly greater ERK kinase activity than wild-type cells after IL-6 depletion for 2 or 3 days. This ERK kinase assay was repeated once with identical results.

After depletion of IL-6 from wild-type cells for 72 hours, readdition of exogenous IL-6 was capable of reinducing phosphorylation of ERK (Figure 2C, right panels), supporting the notion that the RAS-RAF-MEK-ERK pathway was still intact in these IL-6-depleted cells and they were still sufficiently healthy to respond to exogenous stimuli. Analysis from 3 separate experiments showed that phosphorylation of p44 ERK was increased by IL-6 6-fold (\pm 2-fold) and phosphorylation of p42 ERK was increased 3.4-fold (\pm 1-fold). ERK phosphorylation that was constitutive in mutant ras MM cells was completely abolished by the MEK inhibitor PD98059 (Figure 2C, left panels), as was the ERK phosphorylation reinduced in wild-type cells by readdition of IL-6 (Figure 2C, right panel). These results collectively demonstrate that expression of oncogenic ras results in constitutive activation through MEK and ERK in MM cells.

Figure 2. Effect of oncogenic ras expression on activation of ERK in myeloma cells. (A) Empty vector-transfected control cells (C) or mutant ras-transfected cells (mutant N-ras [N] or K-ras [K]) were depleted of IL-6 for 0 hours (day 0), 48 hours (day +2), or 72 hours (day +3), and immunoblot was performed for total ERK (ERK-T) and phosphorylated ERK (ERK-P). Mean expression of phosphorylated ERK from 3 separate experiments is shown at the bottom of the panel, with control cells set arbitrarily at 1. *Significantly different from control value, $P < .05$. (B) ERK in vitro kinase assay performed in which wild-type (WT) cells were compared with mutant N-ras (N) or K-ras (K) cells after 48 or 72 hours of IL-6 depletion. Immunoblot assay shown for expression of phosphorylated ELK-1 (ELK-P) used as an ERK substrate. (C) In left panels, mutant N-ras or K-ras cells were depleted of IL-6 for 48 hours and then treated with or without PD98059 for 2 hours at 10 μ M. In the right panel, wild-type cells (empty vector transfected) were depleted of IL-6 for 48 hours and then treated without IL-6, with IL-6 alone (100 U/mL for 15 minutes), or with IL-6, preceded by 2 hours of PD98059 (10 μ M).



Ras signaling through AKT. The PI3-K/AKT kinase cascade is an additional potential ras-effector pathway.¹¹ This pathway is also critical for MM growth^{15,21,22} and protection against apoptosis.²³ Thus, a similar experimental design was used to test ras-dependent AKT function in the ANBL6 cell lines. As shown in Figure 3A, after 48 and 72 hours of IL-6 depletion (day +2 and day +3), expression of AKT-T was slightly greater in control cells (C) when compared with oncogenic N-ras (N) or K-ras (K) cells. In spite of this, the expression of phosphorylated or activated AKT in both mutant ras-containing cells was higher than control cells at both 48 and 72 hours (Figure 3A). The mean relative expression of phosphorylated AKT (n = 3) is shown below the blot. At both time points, the differences in expression of phosphorylated AKT were

statistically significant ($P < .05$) in comparisons of either N-ras cells with wild-type cells or K-ras cells with wild-type cells. To further confirm a mutant ras-dependent AKT activation, we performed in vitro AKT kinase assays after 72 hours of IL-6 depletion in which AKT was immunoprecipitated and tested for its ability to phosphorylate the GSK-3 substrate. In 2 separate experiments, IL-6-depleted mutant N-ras-expressing MM cells demonstrated considerably greater AKT kinase activity compared with wild-type (WT)-containing cells (Figure 3B, left panel), as did mutant K-ras-expressing MM cells (Figure 3B, middle panel). Even though there was minimal AKT kinase activity in these IL-6-depleted wild-type MM cells, readdition of IL-6 resulted in reactivation of the kinase (Figure 3B, right panel). As shown in

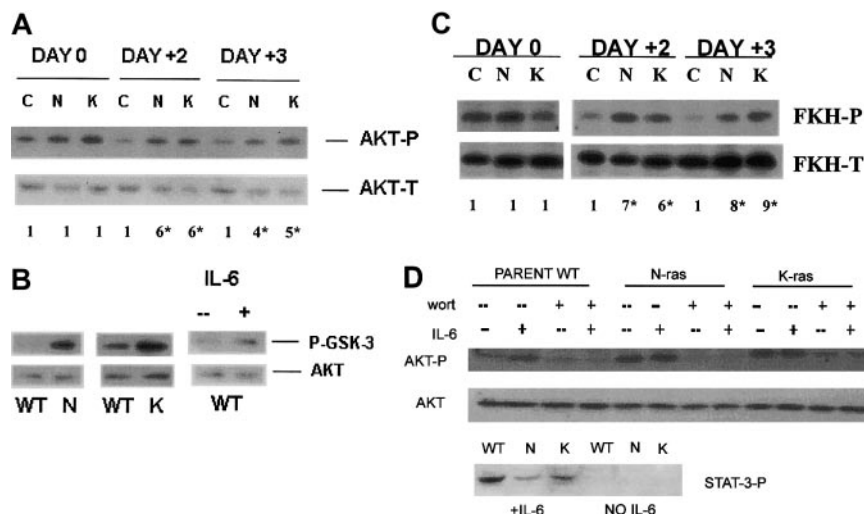


Figure 3. Effect of oncogenic ras expression on activation of PI3kinase/AKT in myeloma cells. (A) Wild-type control (C), mutant N-ras (N), or K-ras (K) cells were depleted of IL-6 as described in Figure 2A, and immunoblot was performed for total AKT (AKT-T) and phosphorylated AKT (AKT-P). Mean expression of phosphorylated AKT from 3 separate experiments is shown at the bottom of the panel, with control (C) cells set arbitrarily at 1. *Significantly different from control value, $P < .05$. (B) AKT in vitro kinase assay performed in separate experiments in which wild-type (WT) cells were compared with either mutant N-ras (N) or K-ras (K) cells after 48 hours of IL-6 depletion. Wild-type cells were also depleted of IL-6 (48 hours) and then treated with or without IL-6 (100 U/mL) for 15 minutes. Immunoblot assay shown for expression of total AKT in immunoprecipitates (AKT) and phosphorylated GSK-3 (P-GSK-3) used as AKT substrate. (C) Three cell lines depleted of IL-6 as in panel A, and immunoblot performed for total forkhead transcription factor (FKH-T) and phosphorylated FKH (FKH-P). Relative mean expression levels of phosphorylated FKH from 4 separate experiments shown at the bottom of the panel. *Significantly different from control (C) value, $P < .05$. (D) Upper panels: wild-type, mutant N-ras, and K-ras cells were depleted of IL-6 for 48 hours and then treated with or without wortmannin (wort) (0.1 μ M) for 2 hours and with or without IL-6 (100 U/mL) for 15 minutes. Immunoblot was then performed for total AKT and phosphorylated AKT. In the lower panel, the same cells (wild-type [WT], mutant N-ras [N], or K-ras [K]) were either continuously cultured in IL-6 (+IL-6) or depleted of IL-6 (no IL-6) for 48 hours. They were then harvested, and immunoblot was performed for tyrosine-phosphorylated STAT-3 (expression of total STAT-3 was comparable in all 6 lanes; not shown).

Figure 3B, comparable amounts of total AKT were contained in these immunoprecipitates.

To test if AKT activation in these MM cells resulted in downstream signaling, we also examined the forkhead transcription factor (FKH), which serves as an AKT substrate.²⁴ Although comparable amounts of total FKH were expressed in all 3 cell lines at 48 and 72 hours after IL-6 depletion, expression of phosphorylated FKH was greater in both mutant ras-containing cell lines when compared with control (C) wild-type myeloma cells (Figure 3C). Densitometric analysis of 4 separate experiments (mean expression shown below the blot) determined that these differences at both time points are significant ($P < .05$).

To test if ras-induced AKT activation was dependent upon preceding PI3-K activation, we used the PI3-K inhibitor wortmannin. Wild-type-expressing and mutant ras-expressing cells were again depleted of IL-6 for 3 days and pretreated with or without wortmannin followed by re-exposure to IL-6. As shown in Figure 3D, once again, after depletion of IL-6 from all 3 lines, while AKT becomes dephosphorylated in the wild-type (parent WT) cell line, its phosphorylation is maintained in both mutant ras-expressing cell lines. In this particular experiment, expression of total AKT was comparable in all 3 cell lines. In addition, re-exposure to IL-6 could reinduce AKT phosphorylation in wild-type cells in a wortmannin-sensitive fashion. Moreover, the constitutively maintained AKT phosphorylation in the mutation-containing cells was also sensitive to wortmannin. These data indicate the PI3-K/AKT cascade is a ras effector in these mutant ras-containing MM cells. In contrast to these results, Figure 3D also demonstrates that tyrosine phosphorylation of STAT-3 is not ras dependent. As shown, tyrosine dephosphorylation of STAT-3 occurred equally in all 3 lines when they were depleted of IL-6. This experiment was repeated 2 additional times, and tyrosine phosphorylation of STAT-3 was consistently abrogated by IL-6 depletion in all 3 cell lines in each experiment. These latter STAT-3 results argue against the possibility that differences in signaling between IL-6-depleted wild-type- and mutant ras-containing MM cells are due to nonspecific differences in the general health of the cells.

Ras signaling to mTOR-p70S6K. Activity of the p70S6 kinase (p70S6K) is required for growth factor-induced expression of ribosomal proteins and elongation factors²⁵ and ultimately for allowing the enhanced translation of transcripts required for cell cycle transit. We have previously shown that induction of p70S6K activity was critical for IL-6-induced myeloma cell growth.²⁶ Furthermore, as pathways involving ERK and PI3-K are known upstream activators of p70S6K and these pathways were constitutively active in mutant ras-containing MM cells, it was certainly

possible that p70 might also be constitutively activated. The p70 activation is mediated by hierarchical phosphorylation at multiple sites. Initial phosphorylation at serine 411 (Ser411), threonine 421 (Thr421) and serine 424 (Ser424) in the autoinhibitory domain relieves pseudosubstrate suppression and facilitates subsequent phosphorylation.²⁷ Threonine 389 (Thr389) phosphorylation then facilitates phosphorylation at threonine 229 (Thr229).²⁸ The critical phosphorylation at Thr389 appears to be mediated by a PI3-K-dependent pathway and the mammalian target of rapamycin (mTOR)^{29,30} while ERK kinases may play a role in phosphorylation of Thr421/Ser424. These sequential events result in optimal activation of p70S6K enzymatic activity.

The 3 ANBL-6 cell lines were depleted of IL-6 and studied for p70S6K phosphorylation on different residues 2 and 3 days later. As shown in Figure 4A, the expression of total p70 was not different between the 3 cell lines nor was it altered upon IL-6 depletion for 48 hours (day +2) or 72 hours (day +3). Furthermore, phosphorylation of p70 at Ser411 was not affected by IL-6 depletion. However, while threonine 389 and threonine 421/serine 424 became dephosphorylated in control, wild-type-expressing MM cells, their phosphorylation was constitutively maintained in both mutant ras-expressing cells. Densitometric analysis of 3 separate experiments determined that these differences in Thr389 and Thr421/Ser424 phosphorylation were statistically significant ($P < .05$) at both time points for both N-ras and K-ras cells. These data suggest that mutant ras maintains constitutive phosphorylation of p70 at Thr389 and Thr421/Ser424. Figure 4B demonstrates, in 1 of 3 separate experiments, that readdition of IL-6 to wild type-expressing cells at day +3 reinduces phosphorylation of p70 on Thr389 and Thr421/Ser424, but has no effect on phosphorylation of Ser411. Thus, phosphorylation of p70 at Ser411 is unaffected by IL-6 and appears constitutive in all 3 cell lines irrespective of ras status.

The constitutive phosphorylation of p70S6K on Thr389 and Thr421/Ser424 in mutant N-ras- or K-ras-expressing MM cells is mediated by upstream activation from ERK- and PI3-K-containing cascades. As shown in Figure 4C, the PI3-K inhibitor LY294002 curtailed constitutive Thr389 phosphorylation in both cell lines but had no effect on phosphorylation at Thr421/Ser424. The MEK-ERK inhibitor PD98059 inhibited constitutive phosphorylation at both Thr389 and Thr421/Ser424. As expected, constitutive phosphorylation at Ser411 was unaffected by either of these inhibitors. These data are consistent with previous work in other cell models^{27,28} and support a scenario where ERK kinase activity induces phosphorylation of Thr421/Ser424, which then allows subsequent PI3-kinase-dependent phosphorylation at Thr389. It is

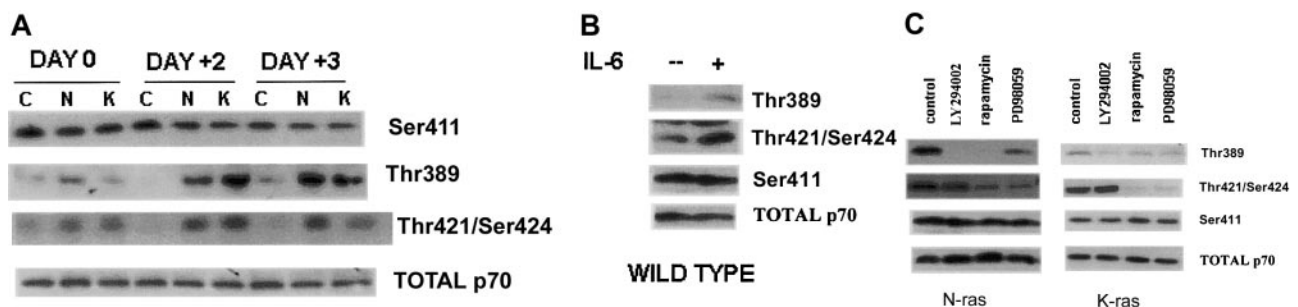


Figure 4. Effect of oncogenic ras expression on p70S6 kinase in myeloma cells. Expression of oncogenic ras results in constitutive phosphorylation of p70S6 kinase in myeloma cells. (A) Control (C; empty vector-transfected), mutant N-ras (N), or K-ras (K) cells depleted of IL-6 for 0, 48, or 72 hours and immunoblot assay performed for total p70S6 kinase (bottom panel) or p70 phosphorylated on serine 411 (Ser411), threonine 389 (Thr389), or threonine 421/serine 424 (Thr421/Ser424). (B) Control cells (wild-type) depleted of IL-6 for 48 hours and then re-treated with or without IL-6 (100 U/mL) for 15 minutes. Immunoblot assay was then performed with the same antibodies as in panel A. (C) Mutant N-ras or K-ras cells were depleted of IL-6 for 48 hours and treated with or without LY294002 (10 μ M for 2 hours), rapamycin (10 nM for 6 hours), or PD98059 (10 μ M for 2 hours); then immunoblot assay was performed with same antibodies as in panels A and B.

unclear how Ser411 becomes phosphorylated, but it does not appear mediated by a ras-dependent pathway. To test whether p70 phosphorylation is mediated by mTOR, we used the mTOR inhibitor rapamycin. As shown (Figure 4C), it inhibited phosphorylation at Thr389 as well as Thr421/Ser424, indicating that ras-dependent ERK and/or PI3-K-induced signaling must flow through mTOR for p70S6K phosphorylation. This experiment using LY294002, rapamycin, and PD98059 as inhibitors was repeated once, with identical results for both N-ras and K-ras cell lines.

Ras signaling through NF-kappaB. Since the NF-kappaB pathway can be an important antiapoptotic pathway in ras-transformed cells,^{13,31,32} it could play a role in IL-6 independence identified in our mutant ras-transfected ANBL-6 MM cells. Furthermore, some preliminary evidence suggests NF-kappaB activity is important in determining survival versus death responses in myeloma cells.^{33,34} In addition to its known antiapoptotic effects, the NF-kappaB pathway can stimulate proliferative potential by inducing expression of important cell cycle proteins like cyclin D1.³⁵ Following IL-6 depletion for 48 hours, the 3 ANBL-6 cell lines were tested for basal NF-kappaB activation by EMSA using the kappaB-binding response element. Figure 5A (left panels) depicts 2 of 4 separate EMSA experiments that consistently demonstrated higher constitutive NF-kappaB activation in mutant N-ras (N)- and K-ras (K)-containing myeloma cells when compared with wild-type (WT)-expressing cells. The 2 kappaB-binding complexes consistently demonstrated in mutated ras-containing cells by EMSAs were specific for NF-kappaB members, as shown by cold competition experiments using wild-type and mutant kappaB probes (Figure 5B). To identify the NF-kappaB family members present in these complexes, we used anti-p65 and anti-p50 antibodies. As shown in Figure 5B for mutant N-ras-containing myeloma cells, although an isotype-identical control antibody had no effect (first lane), both anti-p50 and anti-p65 antibodies induced a supershift. Similar results were seen when nuclear extracts were obtained from mutant K-ras-containing myeloma cells (not shown). In addition, antibodies to p52 and c-Rel had no effect on the DNA-binding complexes. These data demonstrate that the increased DNA-binding complexes in mutant ras-containing cells consist of p65/p50 heterodimers and p50/p50 homodimers.

In the wild-type cells, reintroduction of IL-6 at 48 hours successfully reactivated NF-kappaB activity, as demonstrated by

an increase in kB binding (Figure 5A, right panel). Although the IL-6 effect in wild-type cells was modest, it was consistently present in 4 separate experiments, 1.7-fold \pm 0.3-fold increase in p65/p50 binding and 1.6-fold \pm 0.2-fold increase in p50/p50 complex binding, significantly different from control (no IL-6) ($P < .05$).

Since p65/p50 heterodimers are transcriptionally active, we hypothesized that NF-kB-induced gene expression should also be significantly enhanced in mutant ras-containing myeloma cells. In reporter gene studies, we were able to transiently transfect a kappaB-luciferase (firefly) reporter construct into wild-type ras- and mutant N-ras-expressing ANBL6 myeloma cells. As an internal control for mutant ras-induced effects on the basal promoter, we concurrently transfected the identical construct except that it lacked the 5 tandem-repeat kB-binding elements. Mutant K-ras-containing cells could not be studied owing to very poor transfection efficiencies. Reporter gene expression was normalized for transfection efficiency by cotransfecting a second reporter construct that constitutively expresses *Renilla* luciferase under regulation of the SV40 promoter/enhancer. As shown in Figure 5C, when myeloma cells were studied after 48 hours of IL-6 depletion, mutant N-ras-containing cells demonstrated a 15-fold greater NF-kappaB induced gene expression compared with wild-type-expressing cells. This was specific for kB-driven reporter expression, as no significant difference was demonstrated with the reporter construct that lacked kB-binding elements.

Effects of selective pathway inhibition on cell growth

Since the constitutively active signal pathways in the myeloma lines containing mutated ras may account for the described growth advantage in these cells, these cascades could be excellent targets for therapy. Thus, we initially screened the potential efficacy of such strategies by targeting these signal pathways with inhibitors or by gene transfer.

In these experiments, we directly targeted mutant ras by using farnesyl transferase inhibitors (FTIs); the mTOR/p70S6K pathway by using rapamycin (RAP) or its newly developed analog, CCI-779; the MEK/ERK pathway by using PD98059 (PD); and the PI3-kinase/AKT pathway by using LY294002. To target NF-kB, we acutely expressed the IKB superrepressor gene (IKB-sr), whose encoded protein contains serine-to-alanine mutations that prevent

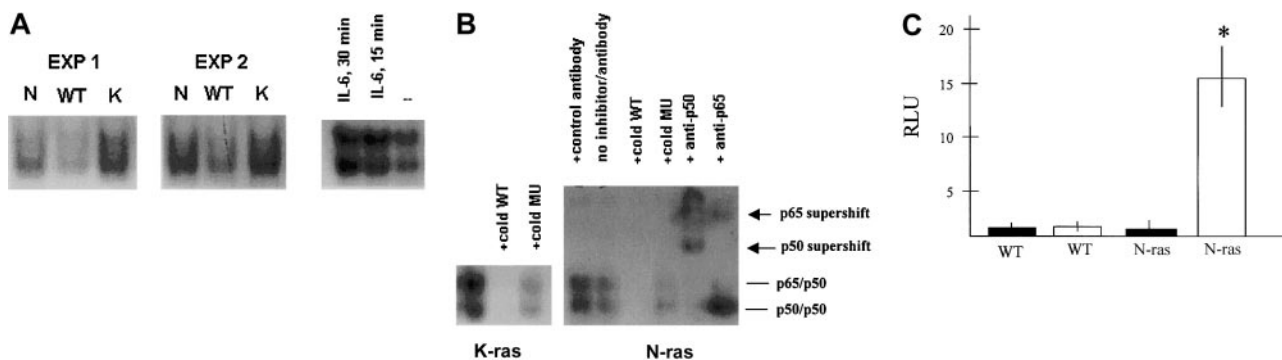


Figure 5. Effect of oncogenic ras expression on NF-kB in myeloma cells. (A) Two separate experiments (left panels) in which EMSA was performed on control, wild-type ras-expressing (WT), mutant N-ras (N) or K-ras (K) cells after 48 hours of IL-6 depletion with the use of the kB-binding DNA oligonucleotide. Wild-type cells were also depleted of IL-6 for 48 hours and then re-exposed to IL-6 (100 U/mL) for 15 or 30 minutes prior to EMSA (right panel). (B) EMSAs performed on mutant K-ras or N-ras cells after 48 hours of IL-6 depletion. With K-ras nuclear extracts (left panel), cold competition was performed with 100-fold molar excess of unlabeled wild-type (cold WT) or mutant (cold MU) kB oligonucleotides. With N-ras nuclear extracts (right panel), similar cold competition experiments as well as supershift experiments were performed. Supershift experiments were performed as described in "Materials and methods" with control antibody or anti-p50 or anti-p65 antibodies. (C) The kappaB reporter expression was analyzed for wild-type (WT)- and mutant N-ras (N-ras)-expressing cells after 48 hours of IL-6 depletion. White open bars (□) are cells transfected with p5x-kB-luc, and black bars (■) are cells transfected with a plasmid that lacked the 5 kB-binding elements. Data are shown as relative light units (RLUs), means \pm SDs from 3 separate experiments after normalization for transfection efficiency. *Significantly different from corresponding wild-type group, $P < .05$.

its phosphorylation and dissociation from NF-kappaB.¹⁶ By preventing dissociation, NF-kB is unable to translocate to the nucleus and induce gene expression. Preliminary experiments demonstrated that infection with an adenovirus expressing IKappaB-sr (or the control virus lacking the transgene, Ad-CMV) successfully transduced more than 80% of mutant N-ras-containing cells at MOIs of 50:1 or greater. The transfection efficiencies for K-ras and wild-type cells were much lower, precluding their testing.

The concentrations of these inhibitor drugs and the IKB-sr adenovirus used in these experiments effectively decreased the activity of their intended targets. As described above, 10 μ M LY294002 and 10 μ M rapamycin prevented p70S6-kinase phosphorylation in mutant ras-containing cells (Figure 4C), and 50 μ M PD98059 inhibited ERK phosphorylation (Figure 2C). Furthermore, Figure 6 demonstrates that FTI-277 effectively blocks ras-dependent induction of ERK and p70 phosphorylation (Figure 6A) in mutated N-ras-containing cells. This concentration of FTI-277 had no effect on phosphorylation of ERK in mutant K-ras cells (not shown). Figure 6B also demonstrates the ability of the IKB-sr to prevent ras-dependent NF-kappaB activation in N-ras cells by gel shift assay (predominantly inhibiting p65/p50 heterodimer binding to DNA).

In initial experiments, the 3 ANBL6 cell lines were depleted of IL-6 and initially exposed to inhibitor drugs for 48 hours in MTT assays. Over a 48-hour time period, the growth of control untreated wild-type ANBL6 cells was not significantly different from growth of untreated mutant ras-containing cells. The concentrations of inhibitor drug solvents were always lower than 0.1% in these assays. Furthermore, separate control wells, in which cells were exposed to the highest concentrations of solvent used, were always

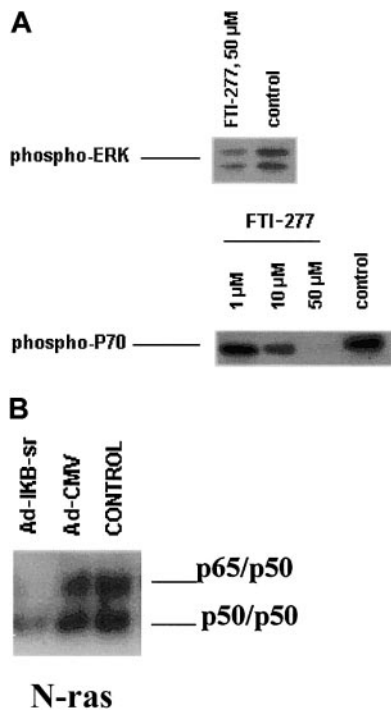


Figure 6. Effect of inhibitors and IKB-sr on phosphorylation of ERK and p70S6 kinase and on NF-kB activation. (A) Mutant N-ras cells were depleted of IL-6 for 48 hours and then incubated without (control) or with FTI-277 at varying concentrations for 24 additional hours. Immunoblot assay was then performed for phosphorylated ERK (top) and phosphorylated p70S6 kinase on threonine 389 (bottom). Immunoblot assay for total ERK and p70 demonstrated equal expression in all lanes (not shown). (B) EMSA experiment shown for IL-6-depleted (48 hours) mutant N-ras cells either not infected (control) or infected with adenovirus expressing IKB-sr (Ad-IKB-sr) or empty vector (Ad-CMV) at an MOI of 50.

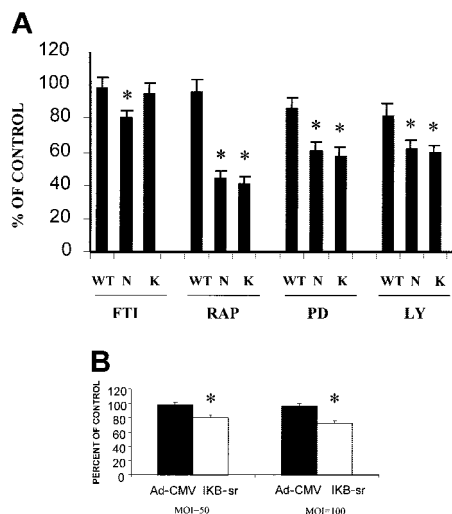


Figure 7. Effect of inhibitors on myeloma cell growth. (A) Wild-type ras-expressing control (WT) or mutant N-ras (N) and K-ras (K) cells incubated without IL-6 and with or without the following inhibitors: farnesyl transferase inhibitor FTI-277 (FTI) at 50 μ M, rapamycin (RAP) at 10 nM, PD98059 (PD) at 50 μ M, or LY294002 (LY) at 10 μ M. MTT assay was performed after 48 hours. Data are the percentage of control (compared with cells incubated without inhibitors), means \pm SDs of 3 to 5 separate experiments for each inhibitor. *Significantly lower than effects on wild-type cells (WT), $P < .05$. (B) Mutant N-ras-containing cells were infected with control adenovirus (Ad-CMV) or virus expressing the IKB superrepressor (IKB-sr) at MOIs of 50 and 100. MTT assays were performed 48 hours later with comparison with noninfected cells. Data are means \pm SDs of 3 separate experiments. *Significantly different from Ad-CMV group, $P < .05$.

included and never showed any toxic effects. As shown in Figure 7A, the farnesyl transferase inhibitor FTI-277 had a very modest selective effect on mutant N-ras-containing cells but no effect against wild-type or mutant K-ras cells. A similar pattern of results was seen with a second FTI, FPT III. At a concentration of 5 μ M, FPT III induced a 15% decrease in survival of mutant N-ras-containing cells, but had no effect on WT or K-ras cells. In contrast, the mTOR inhibitor rapamycin selectively inhibited growth of both mutant ras-containing cells with little effect on wild-type cells (Figure 7A). In experiments not shown, the rapamycin analog CCI-779 had an identical effect at the same drug concentrations. There were also selective effects on mutation-containing cells when the MEK/ERK inhibitor PD98059 and the PI3-kinase inhibitor LY294002 were used, although the inhibitory effects were less impressive (Figure 7A). As shown in Figure 7B, IKB-sr-expressing cells in which p65/p50 binding to DNA was completely blocked also demonstrated a modest inhibition of growth of mutant N-ras-containing cells (Figure 7B).

The cytoreductive effects of FTI-277, rapamycin, PD98059, and LY294002 were all minimal at 24 hours and optimal at 48 hours of incubation. They were not increased further by more prolonged drug exposure. Furthermore, the inhibitory effects were dose dependent. For FTI-277, 15 and 50 μ M induced significant effects of 12% and 18% inhibition, respectively. For PD98059, 10 to 50 μ M was optimal and 0.1 μ M was without effect. With LY294002, 5 to 10 μ M was optimal; 1 μ M was borderline; and 0.1 μ M had no effect. As rapamycin was relatively more effective than the other inhibitors and the selectivity for mutant ras-containing targets more obvious, we used a broad range of concentrations to further test this drug's efficacy. As shown in Figure 8, ID₅₀'s for mutant N-ras- and K-ras-containing cells were approximately 1 nM, while 50% reduction in wild-type cell survival was never reached.

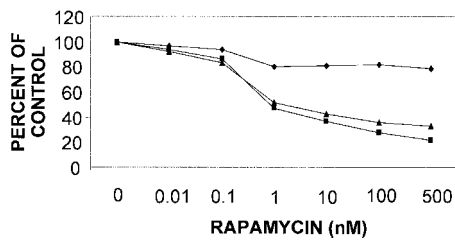


Figure 8. Sensitivity of myeloma cells to rapamycin. Wild-type ras-expressing control cells (♦) or mutated N-ras (▲) or K-ras (■) cells were incubated for 48 hours with 0 to 500 nM rapamycin, and then MTT assays were performed. Data are means of 3 separate experiments (SDs were always less than 5% of the means). Rapamycin-induced inhibition was significantly greater ($P < .05$) for N-ras and K-ras cells compared with wild-type cells at concentrations of 1, 10, 100, and 500 nM.

Cell-cycle analysis was performed to further investigate the cytoreductive effect of FTIs, rapamycin, and the IKB-sr. As shown in Figure 9, the cytoreductive effects of FTI-277 and rapamycin were that of G₁ arrest. Following exposure of mutant N-ras-containing cells to 20 μM FTI-277, the percentage of cells in S phase significantly ($P < .05$) decreased from 19 ± 3 to 11 ± 2. There was no significant increase in apoptosis, as determined by the absence of a sub-G₁ peak. Annexin-V staining was consistent in not demonstrating any FTI-induced apoptosis (Figure 9). In contrast, cell cycle analysis and annexin-V staining successfully identified apoptosis induced in these cells after 24 hours' incubation with anti-fas antibody used at 0.1 μg/mL (17% ± 3% apoptosis by sub-G₁ peak analysis [Figure 9] and 22% ± 4% by annexin-V staining [not shown]). In concurrence with the MTT assays (Figure 7), FTI-277 had no effect on S-phase distribution in mutant K-ras-containing cells (Figure 9). Interestingly, when FTI exposure was performed in low serum concentrations (0.5% FCS), a marked apoptotic effect was seen, and the sensitivity of mutant N-ras cells was comparable to that of K-ras cells and both were significantly more sensitive than the wild-type cells. The induction of apoptosis in low serum concentrations has been identified in other FTI-treated cell types³⁶ and is the subject of a future report.

As with FTIs, the effect of rapamycin (and CCI-779, not shown) was also one of G₁ arrest (Figure 9). Following exposure to 1 μM rapamycin, the percentage of cells in S phase decreased from 19% to 4% in N-ras cells and from 16% to 5% in K-ras cells. There was no significant induction of apoptosis by sub-G₁ peak analysis (Figure 9) or annexin-V staining (not shown).

Acute expression of the IKB-sr gene, using an MOI of 100:1, significantly ($P < .05$) inhibited S-phase percentage, decreasing it from 21% (in empty vector Ad-CMV-infected cells at the same

MOI [Figure 9]) to 13%. A similar inhibition was demonstrated when an MOI of 50:1 was used, inhibiting S-phase distribution from 22% (in Ad-CMV-infected cells) to 12% (not shown). Although completely abrogating the activation of the transcriptionally active p65/p50 dimer (Figure 6), and inducing G₁ arrest, expression of the IKB-sr resulted in only a small increase in apoptosis in these N-ras cells (Figure 9). There was 2% and 3% apoptosis in cells infected with control Ad-CMV virus at 50 or 100 MOI, respectively, and this increased to 5.3% and 7.9% in cells infected with IKB-sr.

Discussion

Ectopic expression of mutated N- and K-ras genes in the IL-6-dependent ANBL-6 myeloma cell line results in the development of IL-6-independent growth.^{7,8} Since ras is a centrally located GTP-binding protein with several possible downstream signal cascade substrates, it is probable that these activated cascades account for stimulated cytokine-independent growth. Thus, to identify these activated cascades, we compared mutation-containing myeloma cells with their control, wild-type ras-expressing cell counterparts after IL-6 depletion. We examined pathways known to be important in MM cell growth. We were successful in identifying constitutive signaling downstream of ras in these cells through the MEK/ERK, PI3-kinase/AKT, mTOR/p70S6-kinase, and NF-kappaB pathways. These differences in signaling between the cell lines when they are depleted of IL-6 could not be explained by nonspecific inhibitory effects of cytokine depletion on wild-type-expressing cells and maintenance of general health in IL-6-independent mutation-containing cells. First, within the time frame of IL-6 depletion, viability was maintained in wild-type cells and remained comparable to that of mutant ras-containing cells. In addition, all signal pathways could be reactivated by readdition of IL-6 in these cells, attesting to their health and the intactness of the cascades. It is also unlikely that the differences in signaling are simply explained by continual IL-6 production from mutant ras cells with autocrine stimulation through glycoprotein 130 (gp 130). First, anti-IL-6 and anti-IL-6 receptor antibodies did not inhibit growth of these cells. Second, in experiments not shown, conditioned media obtained from IL-6-depleted mutant ras cells could not stimulate any signaling in IL-6-depleted wild-type cells. Third, tyrosine phosphorylation of STAT-3 was clearly IL-6 dependent in all 3 cell lines and yet could not be maintained in mutant ras cells when these were depleted of IL-6 (Figure 3). Thus, these data

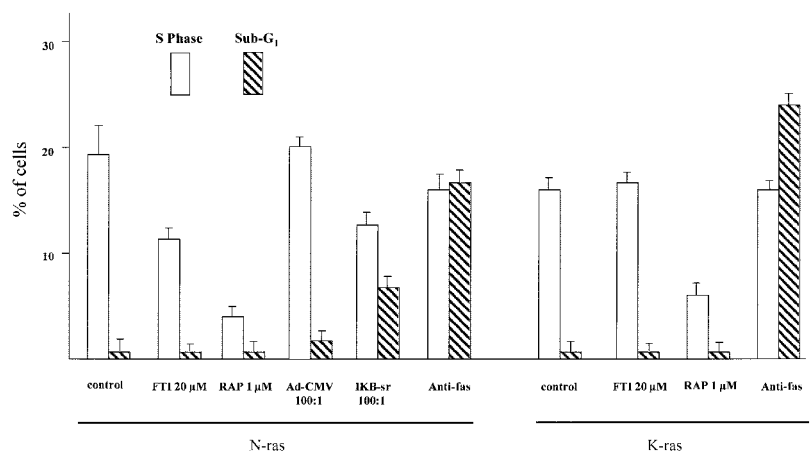


Figure 9. Cell cycle analysis on effects of inhibitors. Cell cycle analysis performed on mutant N-ras- or K-ras-containing cells following treatment for 48 hours without or with FTI-277 at 20 μM, rapamycin (RAP) at 1 μM or anti-fas at 0.1 μg/mL. Analysis was also performed on N-ras cells following infection with control Ad-CMV or IKB-sr adenovirus at MOI = 100. Data represent the percentage of cells in S phase (□) or sub-G₁ fraction (▨), means ± SDs for 3 separate experiments.

strongly argue that oncogenic ras in myeloma cells directly results in downstream signaling through several different cascades but not through STAT-3. It is interesting that STAT-3 activation is not required for continued expansion of ras-mutated myeloma clones. Our data also suggest that the presence of ras mutations in myeloma specimens may account for some of the observed in situ activation of the downstream cascades (like AKT or NF-kappaB)^{21,34} in myeloma bone marrow.

The MEK/ERK and PI3-kinase/AKT cascades are well-known proximal ras substrates. Ras directly activates PI3-kinase through an interaction with the p110 kinase domain of the enzyme,¹¹ and PI3-kinase activity, in turn, results in AKT membrane localization, phosphorylation, and activation.³⁷ Ras also directly interacts with and activates RAF-1, which, in turn, stimulates MEK and, subsequently, ERK kinase activity.³⁸ Activation of these 2 dominant ras-dependent pathways may secondarily account for the stimulation of the p70S6 kinase. The p70 is activated by hierarchical phosphorylation at multiple sites.²⁸ Phosphorylation events at serine 411, threonine 421, serine 424, and threonine 389 synergistically interact to allow penultimate phosphorylation at threonine 229, which results in optimal kinase activity. In mutant ras-containing myeloma cells, a ras-dependent constitutive phosphorylation at threonine 389 and threonine 421/serine 424 was identified in IL-6-depleted cells (Figure 4). In addition, phosphorylation at these residues was IL-6 dependent in wild-type-expressing cells (Figure 4). In mutation-containing cells, phosphorylation at Thr421/Ser424 was mediated by signaling through MEK and ERK, while Thr389 phosphorylation depended on both MEK/ERK- and PI3-kinase-dependent pathways. These results are consistent with the idea that both of these proximal ras-induced cascades interact to optimally activate p70 (identified by Thr389 phosphorylation). In experiments testing in vitro kinase activity of p70 immunoprecipitated from mutant ras cells (not shown), PD98059 and wortmannin both significantly inhibited activity, further supporting this notion. In contrast, phosphorylation at serine 411 appeared to be constitutive, IL-6 independent, and not related to ras status. This could be due to a ras-independent kinase we did not assay that has become constitutively active in wild-type ANBL 6 cells.

NF-kappaB may also be secondarily activated by these ras-dependent proximal cascades. Constitutive activity of the PI3-kinase/AKT pathway may play a role in the increased nuclear accumulation of NF-kappaB in mutant ras cells. As previously published, AKT may interact with IKappaB kinase³⁹ or PI3-kinase may interact with IKB-alpha⁴⁰ to induce NF-kappaB activation. In contrast, ras-dependent activation of NF-kappaB secondary to signaling through MAP-kinase cascades^{13,31,32} appears manifested primarily as a stimulation of the transcriptional function of p65 NF-kappaB rather than induced nuclear localization. In addition, the PI3-kinase/AKT pathway is also capable of independently stimulating the transactivation potential of p65.⁴¹ Thus, it is not surprising that mutant ras expression in myeloma cells results in enhanced NF-kappaB nuclear localization and very significant increased kappaB gene expression.

Our underlying rationale for this study was that identification of specific ras-dependent signaling with constitutive activity would indicate potential explanations for the growth advantage of these mutant clones and suggest molecular targets for future therapy. In that regard, we compared cytoreductive effects on mutation-containing cells with those on wild-type cells, with the idea that differential sensitivities would suggest that an inhibited pathway was specifically important for the growth advantage of mutant ras cells. It is thus gratifying that several inhibitors of these pathways

induced significant effects on mutant ras cells that were significantly greater than on wild-type cells. Since these inhibitors also prevent their targeted signaling in IL-6-stimulated wild-type ANBL 6 cells, they should also be effective in curtailing IL-6-induced growth of wild-type cells. Indeed, preliminary results indicate that rapamycin and FTI-277 inhibit IL-6-induced growth of wild-type cells. It is difficult, however, to quantitatively compare the effects of the inhibitors because of the time constraints imposed on our experiments. Specifically, since growth and viability of wild-type-expressing cells begin to falter after 72 hours of IL-6 depletion, these cells could be fairly compared with mutant ras-containing cells only within the first 3 days of depletion. Thus, it is possible that some pathways that are indispensable for indefinite IL-6-independent growth in mutant ras cells might not be clearly identified within this limited time frame. Nevertheless, differential sensitivities do suggest the constitutive pathways we identified are important for the growth advantage of mutant ras cells.

In 10% FCS, only mutated N-ras-containing cells were sensitive to FTIs. One possible reason for the relative resistance of mutant K-ras is that the affinity of K-ras for farnesyl transferase (FT), the target of FTI, is 10- to 30-fold higher than other forms of ras.⁴² Thus, higher levels of FTIs may be needed to prevent K-ras farnesylation. In contrast, the marked apoptotic response to FTIs in low serum concentration was equally seen on mutant K-ras cells, suggesting a different mechanism. Previous studies⁴³ indicate that low concentrations of serum inhibit AKT activity, and this inhibition may interact with the FTI-induced suppression of ras function to induce apoptosis.

The marked inhibitory effects of the mTOR inhibitors rapamycin and CCI-779 on mutant ras cells are consistent with prior work that demonstrates a correlation with sensitivity and AKT activation.⁴⁴ Thus, ras mutations with attendant heightened AKT activation could be good predictors for successful treatment with these drugs, which are currently in clinical trials. In fact, studies in prostate cancer cell lines demonstrated significant CCI-779 sensitivity in those lines expressing mutated ras.⁴⁴ The inhibition of mTOR and subsequent inhibition of p70S6-kinase activity and 4E-BP1 phosphorylation should prevent translational up-regulation of genes required for cell-cycle transit. This scenario is consistent with our cell cycle analysis, which demonstrated that the mTOR inhibitors induced a G₁ arrest.

Expression of the IKappaB-sr completely abrogated DNA binding of p65/p50 dimers (Figure 6) and induced G₁ arrest, but the induction of apoptosis was less impressive. This was somewhat surprising since, in some previously described models of ras transformation,³¹ enhanced NF-kB activity is a critical determinant of viability in transformed cells. It is possible that infection with adenovirus (to express IKB-sr) artifactually induced additional protective mechanisms that limited apoptosis due to NF-kappaB inhibition. An alternative explanation is that NF-kB activation in myeloma cells may not be a critical antiapoptotic protective mechanism. The previous literature is controversial about this issue. Hideshima et al⁴⁵ reported that the proteasome inhibitor PS-341 inhibited NF-kB activation in myeloma cells and induced apoptosis. However, these investigators found that a more specific NF-kB inhibitor (PS-1145) resulted in modest cytotoxic effects and induction of cell cycle arrest rather than apoptosis.⁴⁶ Thus, at least for those myeloma cells, abrogation of NF-kB activation was not associated with enhanced apoptosis, and the authors concluded that additional effects of PS-341 (not NF-kB inhibition) were responsible for apoptosis. Ni et al³⁴ published similar results, in which

proteasome inhibitors induced a huge amount of apoptosis in myeloma cells in association with NF- κ B inhibition but acute expression of the I κ B- α gene to specifically inhibit NF- κ B induced apoptosis only in 10% of cells above control. Thus, our work is somewhat consistent with that of Ni et al³⁴ and Hideshima et al^{45,46} in that inhibition of basal NF- κ B activation in myeloma

cells induces primarily a hypoproliferative response and apoptosis is enhanced only minimally.

In summary, we have identified several signal pathways in myeloma cells constitutively activated by expression of mutated ras genes. Our results also suggest that these pathways provide excellent molecular targets for future therapy.

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