Leukemic cell growth in the marrow microenvironment may be modulated by stromal cell products, including stimulatory growth factors and the inhibitory regulator prostaglandin E. The production of both of these stromal cell products induced by cytokine mediators appears to be closely linked. Cyclic AMP (cAMP) is an intracellular second messenger that inhibits myeloid cell proliferation and is produced in myeloid leukemia cells on stimulation of adenylate cyclase enzyme by prostaglandin E (PGE). Cells expressing the product of an RAS oncogene have been observed to display diminished hormone-stimulated adenylate cyclase of membranes. If this observation were applicable to myeloid cells, a potentially important mode for leukemia cells expressing p21 RAS to escape inhibitory regulation within the hematopoietic microenvironment would be identified. We studied an interleukin-3 (IL-3)-dependent myeloid cell line, NFS/N1.H7, and a derivative line transfected with H-RAS codon 12 (T24) oncogene, H7 Neo Ras.F3, for inhibition of proliferation by PGE, 1 μM, alone or in combination with pertussis toxin, which inactivates Gi, an inhibitory regulatory guanosine triphosphate (GTP)-binding protein of adenylate cyclase. NFS/N1.H7 cells were inhibited in interleukin-3-dependent proliferation (dose range, IL-3 10 to 100 U/ml) by PGE, 79 ± 11%, by pertussis toxin 51 ± 9%, and by the combination 92 ± 2%, whereas H7 Neo Ras.F3 was inhibited 51 ± 7%, 6 ± 2%, or 58 ± 9% by PGE, pertussis toxin, and the combination, respectively. These differences in capacity for inhibition by adenylate cyclase agonists between RAS-transfected cells (lower inhibition) versus parent cells (greater inhibition) were all highly significant (P < .0005). Intracellular cAMP formed on PGE, stimulation of pertussis-intoxicated cells was 150% lower in RAS-transfected cells than in parent cells. The adenylate cyclase activity of membranes from pertussis-intoxicated RAS-transfected cells was 1.5 to 2 times lower than that of pertussis-intoxicated parent-cell membranes on Mg²⁺-dependent activation by hormone and/or guanine nucleotide. However, very similar adenylate cyclase activity was observed in oncogenic p21 RAS-containing membranes compared with parental membranes under conditions of direct activation by 4 mM Mn²⁺ and forskolin, where inhibitory or stimulatory G-protein influences are minimal. These studies showed diminished adenylate cyclase activity in mutant RAS-bearing myeloid-cell membranes compared with parent-cell membranes independent of the pertussis toxin-sensitive G protein, Gi. This diminished adenylate cyclase activity of membranes is linked to diminished inhibition of cellular response to IL-3 by adenylate cyclase agonists of the p21 RAS-containing cells compared with parental cells and appears to be an important effector function of oncogenic p21 RAS.

**TUMORS MAY ARISE** as a consequence of the action of oncoproteins that code for mutant cellular elements whose normal function is to transmit growth-factor signals within cells. This observation has led to heightened interest in the mechanism of growth-factor signal transduction in various cell types. The vast majority of growth-factor peptides appear to interact with cellular receptors that either possess intrinsic tyrosine kinase activity or that at least propagate tyrosine phosphorylations on cytosolic substrates. However, an early observation showed that induction of a state of cellular competence (for cell-cycle traverse and, hence, proliferation) may also be associated with phosphorylation on cellular substrates by the serine/threonine-specific protein kinase C and subsequent expression of the nuclear c-myc gene product. Activation of the calcium-phospholipid-dependent protein kinase C is normally dependent on initiation of phospholipid metabolism by phospholipase C. In turn, regulation of phospholipase C activation is mediated by guanine nucleotide-binding proteins (G proteins), first recognized as intermediaries between catecholamine, muscarinic, and prostaglandin cell-surface receptors and adenylate cyclase. It was recently proposed that activation of protein kinase C is an integral aspect of the interleukin-3 (IL-3) signal in IL-3-dependent myeloid cells, an observation that implicates guanine nucleotide-binding proteins as possible mediators of IL-3-dependent control of myeloid cell growth.

The association between cellular peptide receptors, guanine nucleotide-binding proteins, and phospholipase C at the plasma membrane has been studied in detail in several model systems but not in IL-3-dependent myeloid cells. In the neutrophil, such studies have demonstrated that the receptor for the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) is physically coupled to the 41-Kd G protein, Gi (named for its inhibitory effect on adenylate cyclase), which serves as the substrate for the bacterial toxin,
pertussis toxin. In its active conformation, this Gi protein also activates phospholipase C, which generates breakdown of phosphatidylinositol 4,5 bisphosphate (PIP2) to yield diacylglycerol, leading to protein kinase C activation and induction of the neutrophil migratory response. Pertussis toxin, which locks Gi in its inactive conformation by covalent modification, ablates these biochemical and functional responses.15,16 These observations suggest that pertussis toxin may also be a useful probe of growth-factor peptide-receptor-mediated proliferation in certain cells, either because of a reliance of their proliferative response upon Gi protein-mediated phospholipase C activation, or, alternatively, by pertussis toxin-mediated reversal of the tonic inhibitory effects of Gi on adenylate cyclase in these cells.17,18 The present study was performed to clarify the role of guanine nucleotide-binding proteins in the control of IL-3-dependent proliferation in myeloid cells, with particular attention to the role of the pertussis toxin-sensitive Gi protein, Gi, and the role of a mutant p21 RAS protein. Our previous studies of myeloid cell lines showed that the role of adenylate cyclase activation by prostaglandin E1 (PGE1) is on cyclic AMP (cAMP)-mediated inhibition of IL-3-dependent proliferation.21 With these data in mind, we studied the effect of pertussis toxin and PGE1, treatment on a naive IL-3-dependent cell line recently generated in this laboratory, NFS/N1.H7, and on a derivative cell line, H7 Neo Ras.F3, transfected with the H-RAS codon 12 (T24) oncogene.20 Primary studies of the naive parent-cell line NFS/N1.H7 revealed the absence of stimulated phospholipid metabolism by the growth factor IL-3.22 The observed inhibition of IL-3-dependent cellular proliferation by pertussis toxin on this cell line could therefore not be explained by inactivation of a Gi protein that functions solely to activate phospholipase C. The loss of effectiveness of pertussis toxin as an inhibitor of IL-3-dependent proliferation in the RAS-transfectant derivative cell line H7 Neo Ras.F3 was studied mechanistically and related to the alternative signaling system relevant to IL-3-dependent proliferation—namely, the adenylate cyclase system. The present study shows that the RAS oncogene product affects IL-3-dependent proliferation, in part, as a result of its inhibition of PGE1 (hormone)-dependent adenylate cyclase activation, and suggests that the importance of p21 RAS oncogene expression in early leukemic cells may be its abrogation of the transmission of prostaglandin-mediated inhibitory signals generated within the hematopoietic microenvironment.

MATERIALS AND METHODS

Murine myeloid cell lines. The fastidious IL-3-dependent cell line NFS/N1.H7 was developed from bone marrow cells of the NFS/N mouse, as described elsewhere.23 This cell line was generated as a model of the fastidious growth requirements of immortal myeloid clones that could develop in the absence of ecotropic type C murine leukemia viruses and in the presence of a paucity of xenotropic and mink cell focus-forming (MCF)-endogenous leukemia viruses, which, if present, might sequentially activate growth-related genes by viral insertional mutagenesis.24 The NFS/N mouse-bone donor strain has severely limited copies of these endogenous murine leukemia virus sequences. IL-3 concentrations of 250 to 500 U/mL were required to attain maximal proliferation of the cell line (data not shown). When washed free of IL-3, these cells also manifested a proliferative response to interleukin-4 (IL-4), as assessed by incorporation of 3H-thymidine in the presence of, but not in the absence of, IL-4.25 The IL-4 response in these cells was not dependent on cellular protein kinase C, as assessed by the failure of cellular protein kinase C depletions to diminish the proliferative response.26 The RAS-containing clones, H7 NeoRas.F3 and H7NeoRas.E5, were generated by electroporation of the NFS/N1.H7 cell line with the vector pAL8, which contains a mutant (codon 12) human H-RAS genomic clone (6.6-kb Bam-HI insert) derived from the T24 bladder carcinoma and driven by an SV40 promoter.27 After electroporation, cells were selected for neomycin resistance (also encoded by the vector) in G418, 1 mg/mL, and subcloned by limiting dilution. Studies of H7 Neo Ras.F3 showed that it contains the 6.6-kb human T24 oncogene and displays constitutive p21 RAS activity in overproduction of 3H-diacylglycerol as compared with parent NFS/N1.H7 cells.28 The RAS-transfected cell lines were shown to express the mutant H-RAS (Val 12) protein by flow cytometric immunofluorescence analysis with a Val 12 RAS-specific monoclonal antibody after fixation of cells with methanol to expose the inner cellular membranes, as described elsewhere29 (data not shown). H7 Neo Ras.F3 and H7 NeoRas.E5 cells were maintained in McCoy's 10% fetal calf serum (FCS) and 10% WEHI-3 CM with G418, 1 mg/mL. These cells were strictly IL-3 dependent, as assessed by their failure to proliferate in the absence of IL-3. Control clones were formed by transfection of NFS/N1.H7 cells with a neomycin-resistance gene-expression vector pNeo.3, as described elsewhere.30

Proliferation assay. Cells were washed free of factor and then plated into assay in triplicate microwells of 96-well flat-bottomed plates at 5 x 104 cells per well in a final volume of 100 mL and incubated overnight at 37°C in 5% CO2. On the following day, each well was pulsed with 0.5 uCi 3H-thymidine, harvested after 6 hours' incubation, and counted by liquid scintillation, as described elsewhere.31 The incubation media consisted of McCoy's 10% FCS with or without (a) 8-bromo-cAMP (8 Br cAMP) (Sigma, St Louis, MO), 0.5 mmol/L (b) PGE1 (Sigma) 10-4 mol/L (1 mL/L), or (c) pertussis toxin, 0.1 to 1,000 ng/mL (List Biologics, Campbell, CA). To these media, purified hematopoietic growth factors were added in a range of concentrations. Purified murine IL-3 and purified recombinant IL-4 were obtained from Genzyme (Boston, MA). Homogenously purified IL-3 was a gift of Dr James N. Ihle, St Jude's Children's Research Hospital, Memphis, TN.

Membrane preparation. Cells were incubated at a concentration of 1 x 106 cells/mL in McCoy's 10% FCS with or without pertussis toxin 0.1 to 100 ng/mL for 4 to 16 hours at 5% CO2, 37°C. Viability, as assessed by trypan-blue exclusion, was not affected by this treatment (greater than 90%). Cells were washed twice in serum-free medium and homogenized in a buffered solution of 15 mmol/L Tris, pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, and 1 mmol/L phenyl methylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 400 g for 10 minutes to remove nuclei, and membranes were isolated by ultracentrifugation (200,000 g for 20 minutes) of the supernatant.

Adenylate cyclase activity assays. Adenylate cyclase reactions were initiated by the addition of 20 mL membrane suspension containing 40 pg protein to a final volume of 100 mL containing 40 mmol/L Tris, 1 mmol/L NaATP, 0.5 mmol/L cAMP, 2.5 U/mL adenosine deaminase, 0.4 mg/mL bovine serum albumin (BSA), 80 mmol/L NaCl, 10 mmol/L MgCl2, and an ATP-regenerating system consisting of 5 mmol/L creatine phosphate and 5 U/mL creatine phosphokinase.29 Reactions were performed to assess generation of 32P-cAMP from 2 mL of 32P-ATP at 30°C for 10 minutes and were terminated by the addition of 0.8 mL of a solution of 0.25% sodium dodecyl sulfate (SDS), 5 mmol/L adenosine triphosphate (ATP), and 0.175 mmol/L cAMP (pH 7.5). The nonhydrolyzable
GTP analogue guanosine 5' (β, γ-imido) triphosphate Gpp(NH)p was used as the stimulus in some reactions in concentrations from 10⁻⁸ to 10⁻⁴ mol/L to assess stimulatory regulatory GTP-binding protein (Gs)-mediated adenylate cyclase activity specifically. Forskolin 10⁻⁴ mol/L was used as the stimulus in other reactions performed with various concentrations of the divalent cations Mg²⁺ or Mn²⁺ in the presence of guanine nucleotides to evaluate inhibitory protein (Gs)-mediated adenylate cyclase activity specifically. Forskolin was used as the stimulus in some reactions in concentrations from 10⁻⁹ to 10⁻⁴ mol/L to assess stimulatory regulatory GTP-binding protein (Gs)-mediated adenylate cyclase activity specifically.

After termination of each reaction, ¹H-cAMP (10⁴ cpm) was added to judge recovery of cAMP after chromatographic isolation by the method of Salomon and colleagues. Each data point represents the mean ± SEM of duplicate reactions.

**ADP ribosylation for SDS polyacrylamide-gel electrophoresis (PAGE) assay of Gi.** The [³²P] ADP-ribosylation reactions were performed by a modification of the procedure described previously. The major substrate of pertussis toxin-catalyzed ADP ribosylation has been previously demonstrated to be Gi, the adenylate cyclase inhibitory GTP-binding protein (41-kD subunit).

**Determination of intracellular ¹H-cAMP generation.** Determination of intracellular ¹H-cAMP was performed according to the methodology of Kienast et al with modification, as described previously. Following preincubation in culture medium with pertussis toxin 100 ng/mL or control medium for 4 hours, cells were suspended at 10⁶ cells/mL in serum-free medium with 3H-adenine 15 μCi/mL for 30 minutes at 20°C. Cells were centrifuged and resuspended in medium at the same cell density. Cell aliquots of 450 μL were added to 50 μL medium containing 3-isobutyl-1-methylxanthine (MIX) 100 μmol/L (final concentration, 10 mmol/L), with or without PGE₂, and incubated at 37°C for 20 minutes. Reactions were stopped by addition of 50 μL 3 mol/L trichloracetic acid containing ¹H-cAMP (ICN Biochemicals, Costa Mesa, CA), 0.2 μCi/mL, vortexed, and then centrifuged at 10,000 × g for 5 minutes. cAMP was isolated from the supernatants by the method of Salomon et al. Results were expressed as the percentage of incorporated ³H-adenine converted to ¹H-cAMP.

**RESULTS**

**Specific inhibition of IL-3-dependent proliferation by pertussis toxin or PGE, is reversed by RAS oncogene.** The capacity of pertussis toxin, which ADP-ribosylates and inactivates a specific guanine nucleotide-binding protein to inhibit proliferation of the parent-cell line NFS/N1.H7, was studied (Fig 1). Over a range of IL-3 concentrations, 10 to 50 U/mL (Fig 1A) or 50 to 250 U/mL (Fig 1B), cellular ³H-thymidine incorporation was approximately 50% inhibited by pertussis toxin (Fig 1). This effect was specific for IL-3-dependent proliferation of NFS/N1.H7 because proliferation of the cell line stimulated by IL-4, 50 to 500 U/mL, was not inhibited by pertussis toxin, at comparable or lower levels of thymidine incorporation (Fig 1). In addition, it was of interest to observe that addition of suboptimal doses of IL-3 and IL-4 in combination resulted in supraadditive proliferation of NFS/N1.H7 (Fig 1). The observation of synergism (supraadditive effect) between the hematopoietic growth factors IL-3 and IL-4 implies different postreceptor signaling mechanisms, which, like inhibition of proliferation by pertussis toxin, serve to distinguish the stimulation by these cytokines.

In order to relate the functional inhibition of IL-3-dependent proliferation to the specific biochemical action of pertussis toxin, dose-response curves for pertussis toxin inhibition of IL-3-dependent proliferation and for ADP ribosylation of cellular G protein were performed (Fig 2). With the concentration of IL-3 held constant, stepwise dilution of pertussis toxin in the proliferation assay revealed the half-maximal effective dose for pertussis toxin inhibition to be approximately 10 ng/mL (Fig 2A). Reduction of ³H-thymidine incorporation of 30,000 to 15,000 cpm, with background proliferation of ≤500 cpm. In parallel, NFS/N1.H7 cells were incubated overnight with pertussis toxin at varying doses, and then membranes were prepared and subjected to in vitro ADP ribosylation with [³²P]-NAD and pertussis toxin activated in vitro with dithiothreitol. These
membranes were then analyzed by SDS-PAGE and autoradiography (Fig 2B). NFS/N1.H7 membranes not intoxicated overnight demonstrated a single intense substrate band at 41 Kd, putative Gi (Fig 2). This band was a specific substrate of ADP-ribosyl transfer by pertussis toxin, because no 32P-ADP was transferred in the presence of 32P-NAD but in the absence of the toxin (control lane, Fig 2B). Increasing doses of pertussis toxin in the overnight intoxication of NFS/N1.H7 increasingly derivatized the 41-Kd substrate, thus preventing 32P-ADP-ribosylation in the in vitro reaction (Fig 2). At 10 ng/mL pertussis toxin overnight, only a small residual capacity for 32P-ADP ribosylation was still present (Fig 2). Therefore, these two dose-response curves appeared to directly relate toxin-induced ADP ribosylation of the 41-Kd protein in vivo with toxin-induced inhibition of DNA synthesis (Fig 2).

One possible consequence of the ribosylation of a 41-Kd G-protein substrate by pertussis toxin was alteration of the basal activity of adenylate cyclase, so that small increases in intracellular cAMP would significantly affect the IL-3-dependent proliferative capacity of NFS/N1.H7. For this hypothesis to hold true, we reasoned that the capacity of the adenylate cyclase agonist PGE1 to inhibit IL-3-dependent proliferation should be high (Fig 3). Indeed, inhibition of IL-3-dependent proliferation in NFS/N1.H7 by 1 μM PGE1 was quite significant and was inversely related in degree to the IL-3 concentration and amount of proliferation (Fig 3).

We also observed that in the RAS-transfectant derivative cell line H7 Neo Ras.F3, the amount of IL-3-dependent proliferation resistant to PGE1 was greater than in NFS/N1.H7 (Fig 3). In order to discount the possibility that the observed greater resistance to PGE1 of the H7 Neo Ras.F3 cell line, compared with parent NFS/N1.H7, was simply due to greater proliferation at a given IL-3 concentration, a series of experiments was performed to individually examine percent inhibition of proliferation of NFS/N1.H7 or H7 Neo Ras.F3 by PGE1 1 μmol/L at a large range of IL-3 concentrations from 10 U/mL to 100 U/mL (Table 1). In fact, the
inhibition of IL-3-dependent proliferation by PGE₁ on NFS/N1.H7 was greater than on H7 NeoRas.F3 (Table 1). More dramatic, however, was the difference between RAS-transfectant and parent cells in antiproliferative efficacy of pertussis toxin on the IL-3 proliferative response. Pertussis toxin did not at all inhibit the RAS transfectant cell line (Table 1), despite the ability for total ADP ribosylation of the 41-kDa substrate in H7 NeoRas.F3 by pertussis intoxication, assessed by SDS-PAGE analysis (data not shown). Consistent with the idea that pertussis toxin and PGE₁ were acting on the same signaling pathway to inhibit IL-3-dependent proliferation, the combination of PGE₁ and pertussis toxin was no more effective in inhibiting H7 NeoRas.F3 but led to almost complete inhibition of NFS/N1.H7 (Table 1). On the other hand, use of the soluble cAMP analogue 8 BrcAMP led to significant and comparable levels of inhibition of both cell lines in response to IL-3 (Table 1).

In other experiments, basal and prostaglandin E₂-generated intracellular cAMP in cells grown in IL-3 were greater in pertussis-intoxicated NFS/N1.H7 cells than in H7 NeoRas.F3 that were also pertussis-intoxicated (Table 2).

Taken together, these data suggested that the p21 RAS-containing cell line was resistant to agents that act through the adenylate cyclase transmembrane-signaling complex consisting of the PGE₁ receptor, the adenylate cyclase catalyst, as well as the regulatory G proteins that inhibit (Gi) or stimulate (Gs) the catalyst. The failure of pertussis intoxication to reverse the resistance of RAS-transfectant cells to inhibition by PGE₁ pointed to another G protein, possibly p21 RAS, as an inhibitory effector responsible for this resistance, or simply a decreased adenylate cyclase catalyst activity in H7 Neo Ras.F3.

Lower adenylate cyclase activity of membranes from H7 NeoRas.F3 compared with NFS/N1.H7 observed in the presence of Me may be corrected by Mn²⁺. To localize the putative defects of PGE₁ or pertussis-toxin augmentation of cAMP generation in H7NeoRas.F3 versus NFS/N1.H7, we measured adenylate cyclase activity of membranes from NFS/N1.H7 and H7 Neo Ras.F3 when stimulated by the hormonal agonist PGE₁ or on stimulation of the catalyst by Gpp NHp-activated Gs (Fig 3). Adenylate cyclase activity of H7 Neo Ras.F3 was always lower than NFS/N1.H7, whether stimulated by PGE₁ or GppNHp (Fig 4, bottom). In addition, pertussis intoxication, which mildly increased adenylate cyclase activity of both NFS/H1.H7 and H7Neo Ras.F3 (Fig 4, top v bottom), did not alter the difference between parent and RAS-transfectant cells (Fig 4, top). We also observed twofold greater adenylate cyclase activity stimulated by hormone or GppNHp in membranes from control neomycin-resistant cells, H7 TKNeo.G9, compared with p21 RAS-containing membranes from H7 Neo.Ras.F3 or the similar RAS-transfectant clone H7 NeoRas.E5 (data not shown).

Differences in adenylate cyclase activity of membranes from the p21 RAS-containing H7 Neo Ras.F3 versus parent NFS/N1.H7 membranes on Mg²⁺-dependent activation of the catalyst by hormone and/or guanine nucleotide ruled out a simple defect of hormone-receptor coupling to Gs in H7 Neo Ras.F3 (Fig 4). To directly assess whether diminished adenylate cyclase activity might be a result of quantitative decrease in amount of catalyst in the membrane, forskolin-

### Table 1. Inhibition of IL-3-Dependent Proliferation of Parent and RAS-Transfectant Cells by PGE₁, Pertussis Toxin, and 8 BrcAMP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Inhibition ± SEM (No. of Determinations)</th>
</tr>
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<tbody>
<tr>
<td>8 BrcAMP (0.5 mmol/L)</td>
<td></td>
</tr>
<tr>
<td>NFS/N1.H7</td>
<td>87.8 ± 2.9% (4)</td>
</tr>
<tr>
<td>H7 NeoRas.F3</td>
<td>81.8 ± 3.9% (4)</td>
</tr>
<tr>
<td>PGE₁ (1 μmol/L)</td>
<td>79.0 ± 11.1% (12)</td>
</tr>
<tr>
<td>Pertussis Toxin</td>
<td>51.2 ± 6.9% (12)</td>
</tr>
<tr>
<td>(100-1,000 ng/mL)</td>
<td>51.2 ± 8.6% (13)</td>
</tr>
<tr>
<td>Pertussis Toxin + PGE₁</td>
<td>92.0 ± 2.0% (8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P value*</th>
<th>≤10⁻⁴</th>
<th>.00041</th>
<th>.00003</th>
</tr>
</thead>
</table>

*Number of determinations at IL-3 dose ranges: 10 to 25 μU/mL, six; 30 to 50 μU/mL, eight; 75 to 100 μU/mL, three. Percent inhibition equals cpm

[^H-thymidine uptake in IL-3 alone, divided by cpm uptake[^H]-thymidine in IL-3 plus inhibitor(s), multiplied by 100.]

[^H-thymidine uptake in IL-3 alone, divided by cpm uptake[^H]-thymidine in IL-3 plus inhibitor(s), multiplied by 100.]

[^P value of Student’s t-test in comparative inhibition of NFS/N1.H7 versus H7 Neo Ras.F3 by the stated agent.]
Table 2. Excess Intracellular cAMP Generation in NFS/N1.H7 Above H7 Neo Ras.F3 After Pertussis Intoxication of Both Cell Types

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Percent cAMP Generated</th>
<th>Fold Stimulation of cAMP Over Basal by PGE&lt;sub&gt;1&lt;/sub&gt; in NFS/N1.H7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>139 ± 18%</td>
<td>—</td>
</tr>
<tr>
<td>0.1 μmol/L PGE&lt;sub&gt;1&lt;/sub&gt;</td>
<td>161 ± 25%</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>1 μmol/L PGE&lt;sub&gt;1&lt;/sub&gt;</td>
<td>146 ± 18%</td>
<td>24 ± 2</td>
</tr>
</tbody>
</table>

Mean ± SEM of two experiments. *Corrected for incorporation of <sup>3</sup>H-adenine. Cells were pertussis-intoxicated for 4 hours at 100 ng/mL of toxin, a maneuver that ADP-ribosylated all detectable G<sub>i</sub>.

stimulated reactions were performed in the presence of a range of Mn<sup>2+</sup> concentrations from 0.5 to 4 mmol/L (Fig 5). Because Mn<sup>2+</sup> independently activates adenylate cyclase at an allosteric site and also differentially uncouples regulatory G proteins from the cyclase catalyst in the concentration range, 0.5 to 4 mM, forskolin/Mn<sup>2+</sup>-stimulated adenylate cyclase reactions were performed without or with added GppNHp (Fig 5). At Mn<sup>2+</sup> concentrations below 2 mmol/L, forskolin-stimulated adenylate cyclase activity was significantly greater in the parent NFS/N1.H7 than in H7Neo Ras.F3, and GppNHp significantly augmented adenylate cyclase activity (Fig 5, bottom, left v right). However, as the Mn<sup>2+</sup> concentration was increased above 2 mmol/L to 4 mmol/L, the difference between the adenylate cyclase activity of H7 Neo Ras.F3 and NFS/N1.H7 was diminished (Fig 5). These observations were also made for membranes from pertussis-intoxicated NFS/N1.H7 and H7 Neo Ras.F3 (Fig 5, top).

DISCUSSION

The present study examined the activity of two inhibitors of myeloid cell proliferation, pertussis toxin and PGE<sub>1</sub>, which exert known effects on elements of transmembrane signaling by hormones and growth factors, for their action in inhibiting IL-3-dependent proliferation of a naive parent-cell line, NFS/N1.H7, and a derivative cell line transfected with the T24 mutant H-RAS oncogene, H7 Neo Ras.F3. These studies were performed to gain insight into the role of an RAS oncogene in a model of neoplastic hematopoietic cell proliferation. Studies in other systems have demonstrated that the most frequent RAS gene mutations in mammalian tumors, including myeloid leukemias, involve codons that give rise to the RAS protein's guanine nucleotide-binding core, like the T24 codon 12 mutation of this study. As a result of these mutations, the intrinsic GTP hydrolytic capacity of p21 RAS is severely impaired, which maintains the effector functions of p21 RAS at the plasma membrane. The first effector function of oncogenic p21 RAS elucidated in model systems was in the overproduction of diacylglycerol (DAG), the primary activator of protein kinase C, perhaps through prolonged phospholipase-C activation at the membrane. The resulting constitutive DAG-mediated protein kinase C activation would relieve certain growth factor requirements for transcription of nuclear protooncogenes that function in initiation of DNA synthesis and mitosis.

More recently, it has been reported that model tumor-cell systems transformed by RAS oncogenes display diminished capacity of hormone-stimulated adenylate cyclase to generate intracellular cAMP. These observations were of interest to us because previous studies from this laboratory had demonstrated that one relevant signal-transduction pathway affecting myeloid-cell proliferation is that involved in generating intracellular cAMP. These studies demonstrated that a sustained increase in intracellular cAMP was a strong antiproliferative signal for both IL-3-dependent and IL-
3–independent myeloid-cell proliferation. Indeed, the relevance of cAMP in IL-3–dependent mitogenesis is probably based in the observation that transcription of at least one gene (c-myc) involved in rendering a state of competence for myeloid-cell proliferation is negatively affected by cAMP-dependent protein kinase A activity.\(^5\) In this regard, p21 RAS would be expected to dampen the inhibitory efficacy of the adenylate cyclase agonist PGE\(_1\) on IL-3–dependent signals.

Thus, another important potential effector function of p21 RAS in myeloid cells besides protein kinase C activation would be on inhibiting signal transduction through the adenylate cyclase system. Indeed, the present study demonstrated resistance to PGE\(_1\)-mediated inhibition of IL-3–dependent proliferation in cells containing the mutant RAS protein (Fig 3; Table 1). This resistance to inhibition of proliferation by PGE\(_1\) was not reversed by pertussis intoxication (Table 1), which covalently modifies the 41-Kd Gi protein that has demonstrated inhibitory activity on the adenylate cyclase system so as to inactivate its inhibitory function.\(^7\) In addition, cells containing mutant p21 RAS formed diminished amounts of intracellular cAMP as compared with their parent cells, a difference not corrected by ADP ribosylation of the 41-Kd G-protein putative Gi by pertussis intoxication (Table 2).

Another view of the significance of pertussis intoxication on inhibiting IL-3–dependent proliferation of the parent NFS/N1.H7 cell line, and its loss of activity in RAS-transfected cells, H7NeoRas.F3, would hold that the signal transduction pathway from the IL-3 receptor couples to the pertussis toxin–sensitive G protein responsible for phospholipase C activation. Peptide receptor–mediated activation of phospholipase C via a pertussis toxin–sensitive G protein has been described.\(^13\)\(^,\)\(^14\)\(^,\)\(^32\)\(^,\)\(^50\) However, our previous investigations of the parent NFS/N1.H7 cell line failed to reveal IL-3–stimulated activation of phospholipase C,\(^26\) which caused us to postulate that the role of inactivation of the 41-Kd Gi by pertussis toxin in inhibiting IL-3–dependent mitogenesis might be on signaling systems other than phospholipase C, such as the adenylate cyclase system. Reports from other laboratories have suggested that p21 RAS may also interact with signaling systems other than phospholipase C to influence growth factor–stimulated mitogenesis.\(^8\) Therefore, in the present study we were particularly receptive to data implicating the adenylate cyclase system in functional responses to pertussis toxin.

Our studies demonstrated inhibition of NFS/N1.H7 parent–cell proliferation by pertussis toxin that was specific for the growth factor IL-3 and that was strictly related to the ADP ribosylation of a single 41-Kd substrate, putative Gi (Figs 1 and 2). The failure of pertussis toxin to inhibit the IL-4 proliferative response in these parent cells may be related to the intrinsic resistance of this growth factor–mediated proliferative response to inhibition by adenylate cyclase agonists, including PGE\(_1\), and to the cAMP analogue 8 BrcAMP (data not shown).

Pertussis toxin was also a useful probe of the effect of p21 RAS on IL-3–dependent proliferation in our study. The absolute loss of pertussis toxin's inhibitory effect on IL-3–dependent proliferation observed in mutant RAS-containing H7 Neo Ras.F3 cells paralleled the diminished efficacy of PGE\(_1\) in inhibiting such proliferation, supporting the concept that these two agents exert their effects on the same signal-transduction pathway (Table 1). The possibility that RAS itself might be an adenylate cyclase inhibitor was strengthened by the failure of pertussis intoxication to reverse resistance of H7 Neo Ras.F3 to PGE\(_1\) (Table 1; PGE\(_1\) plus pertussis toxin).
Our studies of membrane adenylate cyclase activity were divided into those in which activity was absolutely dependent on GTP-dependent signaling (Mg\(^{2+}\) cation-dependent) versus those in which GTP-dependent signals were auxiliary to primary activation of the adenylate cyclase catalyst by forskolin and Mn\(^{2+}\). We reasoned that a capacity for p21 RAS to inhibit adenylate cyclase would be demonstrable in those assays performed in the presence of Mg\(^{2+}\) and guanine nucleotide with or without hormone but might not be observable under certain conditions of direct activation of the catalyst that occur independent of inhibitory influences. We observed significantly lower adenylate cyclase activity in the presence of Mg\(^{2+}\) from membranes of RAS-transfectant H7 NeoRas.F3 cells compared to parent NFS/N1.H7, whether stimulated by hormone or by nonhydrolysable GTP analogue, Gpp(NH)p (Fig 4). Pertussis toxin did not correct the lower adenylate cyclase activity of membranes from p21 RAS-containing cells to the parental level (Fig 4). Such a result could be due either to greater inherent adenylate cyclase activity in parent cells or to the existence of another guanine nucleotide-dependent inhibitory influence in H7 Neo Ras.F3 cells left after inactivation of Gi by pertussis toxin. We therefore compared adenylate cyclase activity of parental NFS/N1.H7 membranes versus p21 RAS-containing H7 Neo Ras.F3 in the presence of Mn\(^{2+}\) and forskolin. In fact, it is known that both forskolin and Mn\(^{2+}\) directly activate adenylate cyclase but that their activation is subject to modulation by the inhibitory (Gi) or stimulatory (Gs) adenylate cyclase regulatory G proteins, except when the Mn\(^{2+}\) concentration is high.\(^{30,31,36,37}\) It has also been observed that forskolin-stimulated adenylate cyclase activity is synergistically augmented by guanine nucleotide (through Gs) in the presence of 0.5 to 2 mmol/L Mn\(^{2+}\), a concentration that uncouples Gi-mediated inhibitory influences.\(^{38}\) However, at high Mn\(^{2+}\) concentrations (eg, 4 mmol/L), augmentation of the forskolin response by guanine nucleotide-activated Gs is diminished because of the Mn\(^{2+}\)-dependent uncoupling of Gs from adenylate cyclase.\(^{39,40}\)

In the present study, forskolin-stimulated membrane adenylate cyclase activity of NFS/N1.H7 was higher than that of H7 Neo Ras.F3 at lower Mn\(^{2+}\) concentrations (0.5 to 2 mmol/L) (Fig 5, left) and was augmented by addition of Gpp(NH)p (Fig 5, left v right). In addition, the difference between adenylate cyclase activity of NFS/N1.H7 and that of H7 Neo Ras.F3 was still observed in membranes from pertussis-intoxicated cells (Fig 5, top). The observation of near-equalization of forskolin-stimulated adenylate cyclase activities of parent and mutant RAS-containing cells with Mn\(^{2+}\) concentrations of 4 mmol/L is consistent with the interpretation that p21 RAS directly mediates adenylate cyclase inhibition, which is GTP-dependent and is uncoupled by Mn\(^{2+}\) (Fig 5).

An indirect mechanism for development of diminished adenylate cyclase activity in mutant RAS-containing cells might be through p21 RAS-stimulated protein kinase C-dependent phosphorylation of adenylate cyclase components. Such "crosstalk" between signaling systems was previously observed in frog erythrocytes treated with phorbol ester and was demonstrated to result from protein kinase C-mediated phosphorylation of the adenylate cyclase catalyst itself.\(^{39}\) On the basis of our previous observation of augmented recovery of \(^{3}H\)-diacylglycerol (like phorbol, a protein kinase C agonist) from the mutant RAS-containing clone H7 NeoRas.F3 compared with parent cells labeled with \(^{3}H\)-glycerol,\(^{26}\) we directly assessed the effect of phorbol (16 to 160 nmol/L TPA) treatment of NFS/N1.H7 on adenylate cyclase activity. Indeed, phorbol ester treatment of parent NFS/N1.H7 reduced adenylate cyclase activity in some experiments, but the reduced adenylate cyclase activity of membranes from phorbol ester–treated cells compared with control membranes was poorly reproducible between experiments and, when present, was not corrected by 4 mmol/L Mn\(^{2+}\) and forskolin (data not shown). We conclude, therefore, that the very reproducible decrease in adenylate cyclase activity we observe in RAS-transfected cells is not purely phosphorylation-dependent. This conclusion is supported by our method for membrane preparation in the absence of phosphatase inhibitors.

Although the exact mechanistic basis for inhibition of adenylate cyclase activity by p21 RAS is subject to debate, the particular importance of adenylate cyclase inhibition as an effector function of mutant RAS in myeloid cells may be a consequence of the paracrine nature of leukemic cell growth in the hematopoietic microenvironment. It has been observed that growth factor–dependent leukemic cells constitutively produce interleukin-1 (IL-1).\(^{39}\) The stimulation of bone marrow microenvironmental cells by IL-1 results in the release not only of growth factors but also of PGE, which exerts an inhibitory influence on myeloid progenitor-cell proliferation.\(^{29,34}\) Recent evidence suggests that RAS oncogene activation may not be the initiating event in leukemic transformation, but rather may confer a further growth advantage to an already transformed but growth factor–dependent and stromal cell-associated clone.\(^{39,56}\) In a paracrine growth model, RAS oncogene activation in the leukemic clone would thus block the growth-regulatory influence of locally produced PGE, allowing for growth advantage over other normal or dysplastic myeloid progenitors (progression).

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