many short similarities several dozen amino acids in length with histone H1 and its variants H1° and H5 (*17, 18*). These similarities typically exhibit 50% identities, and are characterized by matching lysine-rich regions. It is plausible that these similarities reflect regions in the different proteins that are involved in DNA binding. Additional examples of similarities between the yeast enzyme and other proteins include (i) weak similarity (21% identity) between a 125–amino acid region (residues 780 to 905), which includes the putative active site tyrosine at 783, and a stretch in the RNA vesicular stomatitis virus L-protein that is involved in transcription and replication (*19*); (ii) two short stretches (9 and 10 amino acids in length from positions 252 to 260 and 1039 to 1048) with 80% similarity to sequences in the type II regulatory subunit of the cyclic adenosine monophosphate–dependent protein kinase reported to contain a DNA topoisomerase activity (*20*); (iii) a 15–amino acid lysine-rich stretch (residues 327 to 340) with a 67% similarity to a region of human K-*ras* protein (*21*); and (iv) a 25–amino acid stretch with 48% identity with a region of human *myc* protein (*22*). Further biochemical and genetic information on eukaryotic DNA topoisomerase II as well as the other proteins is needed, however, to provide meaningful interpretations of these similarities.

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The Oncogenic Activation of Human p21^{ras} by a Novel Mechanism

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Single amino acid changes were introduced into normal (non-oncogenic) and activated forms of the human H-*ras* protein at a position (residue 116) proposed on structural grounds to represent a contact site with guanine nucleotides. Substitutions at this site could significantly reduce the ability of both forms to bind and hydrolyze guanosine 5′-triphosphate; these substitutions, however, did not necessarily diminish the transforming capacity of activated derivatives. One substitution that severely impairs these functions activated the transforming potential of the otherwise normal polypeptide.

MUTATED VERSIONS OF CELLULAR *ras* genes have been implicated in the development of many human tumors of diverse origin (*1, 2*). These genes encode a polypeptide of 21,000 daltons (p21) that is highly homologous to the transforming protein encoded by Harvey and Kirsten sarcoma viruses. Cellular and viral p21's are membrane-associated polypeptides (*3, 4*) that bind guanine nucleotides (*5*) and possess a weak guanosine 5′-triphosphate (GTP)–hydrolyzing activity (*6, 7*). It has been suggested that *ras* proteins are involved in transmembrane signal transduction—they share structural features with other signal transducers known as G proteins (*8*), and their biological activity may be similarly mediated by GTP binding (*6, 7, 9*). We wished to define residues important for GTP binding in an effort to evaluate the in vivo action of p21's with a reduced affinity for GTP. Comparative analysis of amino acid sequence has revealed several regions of homology between *ras* and other guanine nucleotide–binding proteins, including bacterial elongation and initiation factors, tubulins, members of the G-protein family, and yeast RAS proteins (*2, 8, 10*). One such region of strong homology is represented by residues 110 to 120 of p21 (*10*). This region includes Asn^{116}, a residue predicted on structural grounds to represent a contact site with the pyrimidine ring of guanine (*11*). To directly evaluate the role of this amino acid in the binding of p21 to GTP, we used oligonucleotide-directed mutagenesis to alter human H-*ras* complementary DNA's (cDNA's) at codon-116. We examined the ability of the variant proteins to bind and hydrolyze GTP and promote the morphological transformation of Rat-1 cells.

We replaced Asn^{116} with either of two amino acids: glutamine, representing a conservative substitution, and isoleucine, which would be expected to have a more radical effect on the protein's structure. This was accomplished by converting codon-116 from AAC (asparagine) to CAG (glutamine) or to ATC (isoleucine). The alterations were detected by hybridization to specific oligonucleotide probes and verified by direct analysis of DNA sequence. The mutated cDNA's were recombined with cDNA's containing previously isolated mutations at codon-12 (*6*). In this manner we assembled cDNA's encoding p21's with glycine or valine at position 12, and asparagine, glutamine, or isoleucine at position 116. These cDNA's were introduced into *Escherichia coli* expression vectors as previously described (*6, 12*). To demonstrate the syn-

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thesis of these p21's in *E. coli*, cultures were transformed with the various *ras* expression vectors; transcription of the *ras* cDNA's, under control of the *trp* promoter, was induced by tryptophan depletion (*6*). All vectors directed the synthesis of p21 at levels identical to those described (*6, 12*). To evaluate the ability of these forms of p21 to bind guanine nucleotides, partially purified [>60% homogeneous (*6*)] preparations were incubated with α-labeled [^{32}P]GTP at GTP concentrations of 10^{-9} to $10^{-5}M$. The amount of protein-bound nucleotide was determined by nitrocellulose filter–binding assays (*13*). In close agreement with previous studies (*6, 13*), the concentration of GTP for half-maximal binding was approximately $10^{-8}M$ for p21-VN [p21(Val^{12}, Asn^{116})] and p21-GN [p21(Gly^{12},Asn^{116})] (Fig. 1). Both Gln^{116} forms of p21 (p21-GQ and p21-VQ) exhibited a somewhat reduced affinity (approximately tenfold) for GTP. More striking, however, was the behavior of the Ile^{116} forms of p21 (p21-GI and p21-VI)—these did not detectably bind GTP. When we examined the ability of p21^{ras} present in the soluble fraction of *E. coli* to bind GTP, we obtained qualitatively similar results indicating that the failure of Ile^{166} forms of the protein to bind GTP was not a reflection of their selective inability to properly renature after purification.

To determine the effects of these alterations on the proteins' guanosine triphosphatase (GTPase) activity, we incubated the four forms of the polypeptide with [γ-^{32}P]GTP and monitored the rate of hydrolysis by resolution of the reaction products by means of thin-layer chromatography (*6*). As previously demonstrated (*6, 7, 12, 14*), the ability of p21^{ras} to hydrolyze GTP is reduced approximately tenfold by most known activating substitutions (for example, Val^{12}). This behavior was not significantly affected by the asparagine-to-glutamine substitution at position 116 (Table 1). However, we detected no hydrolysis of GTP with versions of p21 that contained the asparagine-to-isoleucine substitution at this position.

To determine the effects of these amino acid substitutions on the transforming ability of the polypeptides, we introduced H-*ras* cDNA's into mammalian expression vectors. Briefly, the *ras* cDNA's were transferred from the bacterial expression vectors (*6*) into SV40-based vectors (*15*) at a site following the SV40 early promoter; signals necessary for proper messenger RNA processing were provided by appropriate sequences from hepatitis B viral DNA (*16*). The vectors were transfected into Rat-1 cells by $CaPO_4$-mediated DNA precipitation, and foci of cells overgrowing the monolayer were scored 14 to 21 days later. The cellular H-*ras* gene (pH-Ras) did not induce focus formation, while the activated gene derived from T24 bladder carcinoma cells (pT24-Ras) (*1*) efficiently induced the appearance of foci (Table 2). Similarly, SV40-based vectors encoding p21-VN, but not p21-GN, promoted the transformation of Rat-1 cells. Glutamine-116 versions of p21, which have a slightly reduced GTP-binding capacity as compared to the normal (Asn^{116}) protein, functioned as did their normal counterparts in this assay. Surprisingly, replacement of Asn^{116} with Ile^{116}, a substitution that dramatically reduces the ability of the protein to bind GTP, only slightly affected the ability of Val^{12} forms of p21 to transform Rat-1 cells: we obtained similar numbers of foci although they appeared 4 to 7 days later than foci derived from cells transfected with vectors encoding p21-VN. Even more surprisingly, this substitution was found to activate the otherwise nontransforming Gly^{12} form of p21: pSV-Ras(GI) transformed cells with efficiencies similar to that of pSV-Ras(VN). These foci developed a few days later; once detected, they exhibited the same morphology and growth rate as cells transformed by pSV-Ras(VN). Essentially identical results were also obtained with all such altered H-*ras* cDNA's expressed under the control of the promoter in the long terminal repeat of Rous sarcoma virus.

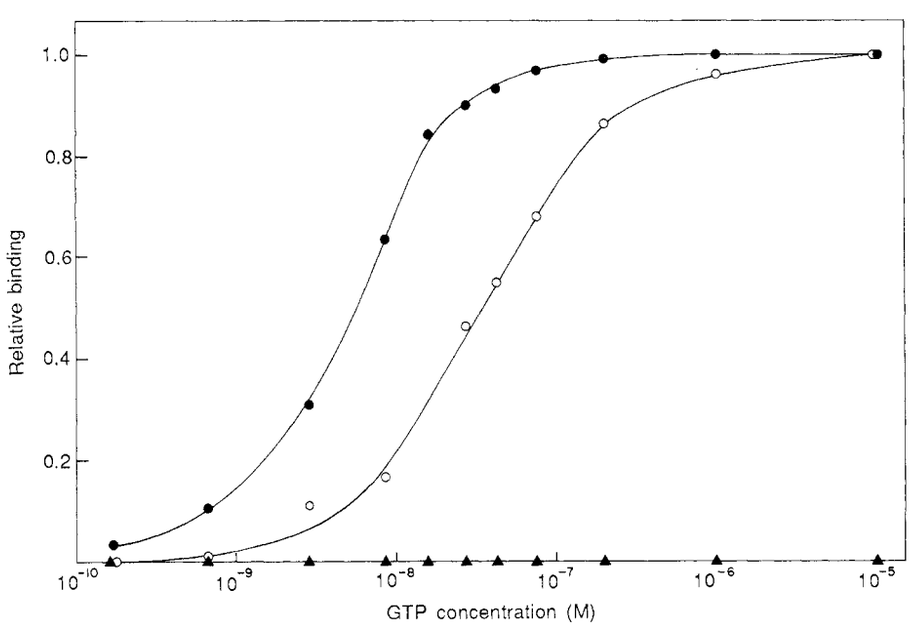

Fig. 1. Binding of GTP by p21. H-*ras* cDNA's encoding p21(Gln^{116}) and p21(Ile^{116}) were obtained by site-directed mutagenesis of M13 clones containing the H-*ras* cDNA (*17*). Oligonucleotide primers used were 5′-CAGGTCACACTTTTGCCCCACCAGC-3′ and 5′-CAGGTCACACTTGATCCCCAC-CAGC-3′, respectively. After verification of the mutant clones by dideoxynucleotide sequencing (*26*), the inserts were recombined into bacterial expression vectors (*6*) and introduced into *E. coli* (*6*). The resulting p21 was partially purified from induced cultures as described (*6, 12*). Ten to 30 ng of p21 was incubated (*12*) in 50 μl of buffer containing the indicated levels of GTP to which [α-^{32}P]GTP was added. The incubation period was 60 minutes at 37°C. The amount of bound GTP was determined by p21 retention on nitrocellulose filters (*13*). Values reported are the average from three separate experiments and represent the derived percentage of p21 bound to nucleotide at various nucleotide concentrations. In all cases, binding of the Asn^{116} forms of the protein (p21-GN and p21-VN) was essentially stoichiometric at all GTP concentrations in excess of $10^{-7}M$. ●, p21-VN; ○, p21-VQ; ▲, p21-VI. In each case, the Gly^{12} version behaved identically to its Val^{12} counterpart.

These results revealed that forms of p21 that are deficient in GTP-binding ability can retain the capacity to induce focus outgrowth of Rat-1 cells. To evaluate the transformation potential of the altered p21's by an additional criterion, we assessed the ability of Rat-1 cells expressing the various p21's to grow in an anchorage-independent fashion. We accordingly cotransfected SV40-based *ras* expression vectors onto Rat-1 cells in the presence of a plasmid encoding neomycin resistance (*17*). Stably transfected cells expressing the *neo* marker were selected by resistance to the antibiotic G418 and were propagated as mass populations. Cells cotransfected with *ras* vectors encoding p21-GN exhibited a normal morphology, whereas vectors encoding p21-VN appear highly transformed. Similarly, cells transfected with plasmids encoding either p21-GI or p21-VI had the typical morphology of highly transformed fibroblasts. Rat-1 cells transfected with the various *ras* vectors were suspended in soft agar medium, and the growth of

colonies was scored 14 days later. Normal Rat-1 cells or Rat-1 cells transfected with a vector encoding the p21-GN form of the *ras* protein were unable to grow, while cells transfected with vectors encoding p21-VN grew efficiently (Table 2). Cells derived from cultures transfected with pSV-Ras(GI) and pSV-Ras(VI) also grew relatively well without anchorage support, providing additional evidence that these forms of $p21^{ras}$ are capable of effecting the transformation of Rat-1 cells.

To document the presence of $p21^{ras}$ in the G418-resistant subclones, cells were labeled with [^{35}S]methionine, and lysates were immunoprecipitated with antiserum to the protein. The immunoprecipitated proteins were resolved by electrophoresis on 12% sodium dodecyl sulfate–polyacrylamide gels (Fig. 2). No p21 was immunoprecipitated by preimmune serum (lane a), while only a faint doublet, presumably reflecting endogenous p21, was immunoprecipitated from normal Rat-1 cells with antiserum to p21 (lane b). In contrast, prominent doublets, corresponding in size to precursor and processed forms of p21 (*4*), were detected in all cultures transfected with the

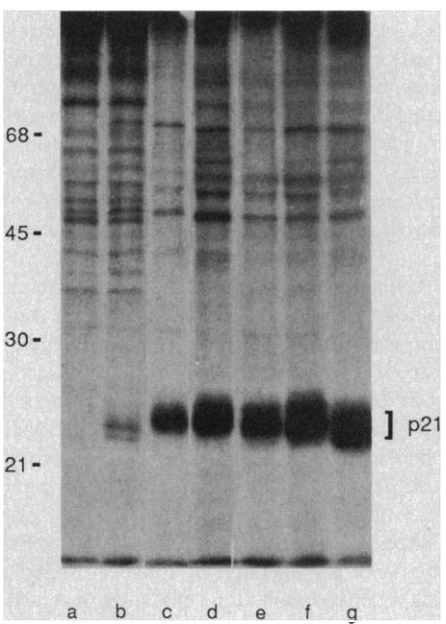

Fig. 2. Expression of p21 in transfected cell lines. Individual subclones of normal and *ras*-transfected Rat-1 cells were labeled with [^{35}S]methionine. Extracts were immunoprecipitated with preimmune rabbit serum (lane a), or antiserum to p21 (lanes b to g) as described (*17*). Extracts were derived from normal Rat-1 cells (lane b) or cells transfected with pSV-Ras(VN) clone 1 (lanes a and c); pSV-Ras(VQ) clone 2 (lane d); pSV-Ras (VI) clone 1 (lane e); pSV-Ras(VI) clone 2 (lane f); pSV-Ras(GI) clone 1 (lane g). Immunoprecipitated proteins were analyzed by electrophoresis on 12% SDS-polyacrylamide gels (*27*) and visualized by fluorography after treatment with En-Hance (New England Nuclear).

ras vectors. Our findings that p21-GI (lane g) exhibits a higher electrophoretic mobility than p21-VI (lane f) extends observations that this position-12 substitution similarly affects the electrophoretic mobility of Asn^{116} forms of the protein (*18*).

Our results confirm predictions that residue 116 plays a critical role in the binding of GTP to the *ras* polypeptide (*11, 19*). These predictions were based on the structure, revealed by x-ray crystallographic analysis, of the *E. coli* bacterial elongation factor EF-Tu (*20*); this polypeptide exhibits a striking homology to p21 in those domains that comprise the guanine nucleotide–binding site (*11, 19*). Specifically, Asn^{116} of p21, by analogy with EF-Tu, is believed to form a hydrogen bond with the O(6) keto group on the pyrimidine ring of guanine (*20*). Why might the transformation potential of activated p21 be relatively unaffected by mutations that drastically reduce GTP-binding affinity, and why might such mutations in and of themselves activate normal p21? By analogy to other signal-transducing proteins whose activity is mediated by the binding of guanine nucleotides (*9*), p21 is thought to be activated as a result of an interaction with a cell surface receptor that promotes the exchange of GTP for guanosine 5′-diphosphate (GDP) (*2*). Deactivation is achieved by the hydrolysis of bound GTP to GDP; an impairment of this reaction is thought to account for the increased transformation potential of p21's activated by typical (for example, position 12) amino acid substitutions (*2, 12*). While it is possible that the Ile^{116} substitution activates p21 by fortuitously eliciting an allosteric change normally promoted by the binding of GTP, we favor an explanation that derives from the altered kinetics of GTP-GDP binding.

Discrimination in the equilibrium binding constant of p21 for guanine nucleotides is achieved by alterations of the kinetic "on" and "off" rates. If, as is probable, the reduced affinity of p21(Ile^{116}) for guanine nucleotides reflects a greatly enhanced rate of dissociation rather than a reduced rate of association, normal mechanisms that prevent the exchange of GTP for GDP will be subverted. Specifically, the usual rate-limiting step for reactivating p21 will no longer be controlled by the interaction of p21 with (for example) an activated cell surface receptor, but rather the dissociation rate of bound GDP. Since the physiological concentrations of GTP and GDP are thought to be in the ratio of 25:1 (*21*), similar binding constants for the two nucleotides will favor formation of the active [p21(GTP)] complex. By this scenario the equilibrium between p21(GTP) and p21(GDP) would shift toward the active [p21(GTP)] species as a result of mutations that reduce the affinity of p21 toward guanine nucleotides. This shift in the steady-state distribution of the two species, we propose, accounts for the transforming ability of the Ile^{116}-substituted protein. Furthermore, insofar as this altered distribution results from a weakened affinity for guanine nucleotides, it illustrates why *ras* proteins exhibit a much higher affinity for guanine nucleotides than is required to ensure near-quantitative binding.

If an Ile^{116} substitution activates the transforming potential of p21, one may ask why naturally occurring activating substitutions are apparently confined to residues 12, 13, and 61 (*1, 2*). It is possible that p21(Ile^{116}) represents a relatively weakly activated protein. Although the biochemical basis of this is open to speculation, it is clear that amino acid substitutions can differ in their potential to activate the transforming potential of p21. For example, p21(Lys^{12}) is a less potent transforming protein than p21(Val^{12})

Table 1. Guanosine triphosphatase (GTPase) activity of altered p21's. Extracts containing 0.2 μg of p21 were incubated with [α-^{32}P]GTP for various times. The products of the reaction were resolved by thin-layer chromatography as described (*6*). Reported values are derived from rates obtained during the linear portion of the reaction.

Protein	Relative rate of hydrolysis
p21-GN	100
p21-VN	12
p21-GQ	80
p21-VQ	21
p21-GI	<0.5
p21-VI	<0.5

Table 2. Transformation potential of altered p21's. The foci were measured in Rat-1 cells that were transfected (*28*) with 10 μg of plasmid DNA's encoding the indicated forms of p21 (*17*). Foci were directly scored after 14 to 21 days. To measure relative plating efficiency Rat-1 cells were cotransfected with the indicated vectors in the presence of a plasmid encoding the bacterial neomycin resistance gene (*17*). G418-resistant cells (10^3 to 10^4) were seeded into 60-mm dishes in medium containing 1% agar. Colonies >30 cells were scored 14 days later. Values are reported relative to the plating efficiency (5.4%) of cells transfected with the vector pT24-Ras. ND, not determined.

Vector	Foci	Relative plating efficiency
pH-Ras	0	<0.001
pT24-Ras	70	1.00
pSV-Ras(GN)	0	<0.001
pSV-Ras(VN)	240	0.83
pSV-Ras(VQ)	80	0.45
pSV-Ras(GQ)	0	ND
pSV-Ras(GI)	160	0.32
pSV-Ras(VI)	45	0.21

(*17*); this difference, however, is only apparent at limiting protein concentrations (*17, 22*). It is possible that transformation by p21(Ile^{116}) represents an in vitro anomaly; that is, this form of the protein may be unable to transform cells by the criterion of malignancy. There is some support for this: substitutions at certain positions (for example, 59 and 63) can be activating by in vitro criteria, but have yet to be found as activating lesions in vivo (*23*). Alternatively, there is compelling evidence that activating lesions promoted by the action of carcinogens occur in a profoundly nonrandom fashion. For example, in 36 animals harboring tumors with position 12–activated H-*ras* genes induced by the action of *N*-nitroso-*N*-methylurea, the same mutation (a G → A transition in the second nucleotide of the 12th codon) accounted for each activation (*24*). As such, it is possible that the majority of "spontaneous" mutations are similarly nonrandom; if so, perhaps nucleotides comprising codon-116 are relatively resistant to the action of those carcinogens to which humans are most typically exposed.

Note added in proof: After submission of this manuscript Sigal *et al.* (*25*) reported that p21^{ras} substituted at position 119 displays reduced affinity toward guanine nucleotides and increased morphological transformation potential.

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Immunoregulatory Feedback Between Interleukin-1 and Glucocorticoid Hormones

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The production and action of immunoregulatory cytokines, including interleukin-1 (IL-1), are inhibited by glucocorticoid hormones in vivo and in vitro. Conversely, glucocorticoid blood levels were increased by factors released by human leukocytes exposed to Newcastle disease virus preparations. This activity was neutralized by an antibody to IL-1. Therefore the capacity of IL-1 to stimulate the pituitary-adrenal axis was tested. Administration of subpyrogenic doses of homogeneous human monocyte–derived IL-1 or the *p*I 7 form of human recombinant IL-1 to mice and rats increased blood levels of adrenocorticotropic hormone (ACTH) and glucocorticoids. Another monokine, tumor necrosis factor, and the lymphokines IL-2 and γ-interferon had no such effects when administered in doses equivalent to or higher than those of IL-1. The stimulatory effect of IL-1 on the pituitary-adrenal axis seemed not to be mediated by the secondary release of products from mature T lymphocytes since IL-1 was endocrinologically active when injected into athymic nude mice. These results strongly support the existence of an immunoregulatory feedback circuit in which IL-1 acts as an afferent and glucocorticoid as an efferent hormonal signal.

INTERLEUKIN-1 (IL-1), A PROTEIN produced predominantly by stimulated macrophages and monocytes, exerts several biological actions. Among the immunological effects of IL-1 are the control of differentiation and activation of lymphocytes and the stimulation of lymphokine production (*1*). IL-1 has also several nonimmunological functions. It acts as an endogenous pyrogen, stimulates hepatocytes to elaborate acute-phase proteins, augments granulocyte superoxide production, and alters fibroblast growth and collagenase and prostaglandin production (*2*). In humans and animals, the production and action of IL-1, of several lymphokines, and of other mediators of inflammation are inhibited by glucocorticoids (*3, 4*). These properties of glucocorticoids explain at least in part their effects on immune and inflammatory responses. The existence of a physiological interaction between adrenocortical and immune cell functions is reflected by the fact that animals undergoing immunological responses to various antigens show, at the same time, increased glucocorticoid blood levels in proportion to the magnitude of the immune response (*5*). Furthermore, incompletely characterized agents derived from activated lymphoid cells have been shown to increase blood levels of glucocorticoids (*6,*

While studying neuroendocrine effects following the inoculation of Newcastle disease virus (NDV) into mice, we observed a marked increase in the blood levels of adrenocorticotropic hormone (ACTH) and corticosterone. This increase was also observed when animals were injected with supernatants derived from cocultures of NDV preparations and either human peripheral blood leukocytes (HPBL) or mouse spleen cells (*8*). Since the injection of appropriate control supernatants had no effect, the increase in ACTH and corticosterone appeared to be induced by a product released from stimulated leukocytes (*9*). Supernatants from cultures of leukocytes exposed to natural infective agents or their products are expected to contain IL-1 (*10*). We therefore treated the human leukocyte–derived, endocrinologically active supernatants with an

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