Blocking Oncogenic Ras Signaling for Cancer Therapy

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The Ras gene product is a monomeric membrane-localized G protein of 21 kd that functions as a molecular switch linking receptor and nonreceptor tyrosine kinase activation to downstream cytoplasmic or nuclear events. Each mammalian cell contains at least three distinct ras proto-oncogenes encoding closely related, but distinct proteins. Activating mutations in these Ras proteins result in constitutive signaling, thereby stimulating cell proliferation and inhibiting apoptosis. Oncogenic mutations in the ras gene are present in approximately 30% of all human cancers. K-ras mutations occur frequently in non-small-cell lung, colorectal, and pancreatic carcinomas; H-ras mutations are common in bladder, kidney, and thyroid carcinomas; N-ras mutations are found in melanoma, hepatocellular carcinoma, and hematologic malignancies. The ras-signaling pathway has attracted considerable attention as a target for anticancer therapy because of its important role in carcinogenesis. In this review, the physiologic and biochemical properties of the Ras proteins, their mechanism of cell signaling, and their relation to human cancer will be discussed. Novel cancer therapeutic approaches based on the inhibition of Ras-mediated signaling, including inhibition of Ras processing, inhibition of Ras protein synthesis, and blockade of downstream Ras effectors, will be discussed. [J Natl Cancer Inst 2001;93:1062–74]

Cell proliferation, differentiation, and survival are regulated by a number of extracellular hormones, growth factors, and cytokines in complex organisms. These molecules serve as ligands for cellular receptors and communicate with the nucleus of the cell through a network of intracellular signaling pathways. In cancer cells, dysregulated cell signaling and proliferation may occur through overexpression or mutation of proto-oncogenes. One such proto-oncogene is ras, which functions as a molecular switch in a large network of signaling pathways, mainly controlling the differentiation or proliferation of cells. Mutated ras genes encode constitutively activated proteins, which have been implicated in tumorogenesis. Mutations in ras have been identified in approximately 30% of all human cancers, making this G protein an important target for the development of anticancer drugs.

METHODS

Original data for inclusion in this review were identified through a MEDLINE® search of the literature. All papers from 1966 through 2000 were identified by use of the following search terms: ras genes, Ras proteins, oncogenic Ras, Ras signaling, and cancer therapy. All original research and review papers related to the role of Ras in oncogenesis and therapeutic interventions relating to Ras signaling were identified. This search was supplemented by a manual search of the proceedings of the annual meetings of the American Association for Cancer Research, the American Society of Clinical Oncology, and the American Association for Cancer Research–European Organization for Research and Treatment of Cancer–National Cancer Institute Symposium on New Anticancer Drugs.

Oncogenic Ras Proteins

In addition to R-ras and M-ras genes, there are three potentially oncogenic ras genes in human cells, which encode four highly related proteins H-ras, N-ras, and K-ras (K4A- and K4B-) (1). The 21-kd transforming proteins of the Harvey (H) and Kirsten (K) murine sarcoma viruses, called v-H-ras and v-K-ras, are oncogenic mutants of normal cellular ras (c-H-ras and c-K-ras). So far, the neuroblastoma (N)-ras has not been found in any retrovirus. The K-ras gene is alternatively spliced, resulting in two protein isoforms, K-rasA and K-rasB. After farnesylation, membrane anchorage of K-rasA occurs through palmitoyl moieties, whereas that of K-rasB occurs through lysine residues.

Ras is a membrane-bound guanosine triphosphate (GTP)/guanosine diphosphate (GDP)-binding (G) protein that serves as a “molecular switch,” converting signals from the cell membrane to the nucleus. These chemical signals lead to protein synthesis and regulation of cell survival, proliferation, and differentiation. However, unlike the classic heterotrimeric G proteins, Ras exists as a monomer. Each Ras protein consists of about 190-amino-acid residues that are highly conserved in the N and C termini. Most of the differences between these proteins occur in the near C-terminal hypervariable domain of about 25 amino acids, which is presumed to be responsible for their different functions. There is a close structural and sequence homology between the monomeric Ras family G proteins and α-subunits of the classic trimeric G proteins (2,3).

The Ras family includes several distinct members, such as Ras (H, K, M, N, and R), Rap (1 and 2), and Ral, that share at least 50% sequence identity. This family shares at least 30% sequence identity with several other small monomeric G protein families, such as the Rho/Rac/CDC42, Rab/Ypt, Ran, Arf, and Rad families (4). Rap proteins are of special interest because they contain the same effector domains as Ras. Rap 1 has been implicated in a number of cellular processes, such as platelet activation and T-cell anergy. The function of Rap 2 remains unclear.

Protein Prenylation

Prenylation is the covalent addition of either farnesyl (15-carbon) or geranylgeranyl (20-carbon) groups to conserved carboxy-terminal cysteine residues of certain proteins (5) (Fig. 1). Prenylation is required for membrane interactions of a number of Ras proteins (6).

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of small hydrophilic proteins involved in cell signaling, including ras, and is catalyzed by three enzymes. The first enzyme, protein farnesyl transferase (FT), transfers a farnesyl group from farnesyl-pyrophosphate (FPP) to the terminal cysteine at the carboxyl end of target proteins. This enzyme recognizes a specific “CAAX” sequence, where “C” represents cysteine, “A” represents an aliphatic amino acid (leucine, isoleucine, or valine), and “X” is methionine, serine, leucine, or glutamine. The second enzyme, protein geranylgeranyl transferase type 1 (GGT-I), transfers a geranylgeranyl group from geranylgeranyl pyrophosphate (GGPP), also to CAAX-containing proteins. The terminal “X” of the CAAX box determines whether farnesylation or geranylgeranylation occurs (6,7). After farnesylation, CAAX proteins undergo two additional steps, cleavage of the “AAX” followed by methylation (8) (Fig. 2). The third enzyme, protein geranylgeranyl transferase type 2 (GGT-II), transfers geranylgeranyl groups from GGPP to proteins containing two carboxy-terminal cysteine (CXC) residues (9,10). Prenylated proteins predominantly contain the CAAX motif and are mostly geranylgeranylated. Farnesylated proteins have attracted attention because farnesylation is critical for oncogenic ras signaling (11). Almost all prenylated proteins that contain the CXC motif (and are substrates for GGT-II) are members of the Rab family. These G proteins regulate intracellular protein secretory pathways (12).

**RAS PROCESSING**

Fig. 2 schematically presents Ras processing. The CAAX box is farnesylated by FT as described above, followed by proteolytic removal of the AAX residues. The exposed carboxyl group of the farnesyl-cysteine is methylated, followed by palmitoylation on the -SH group of the penultimate cysteine residue. This last reaction is reversible and occurs only for Ras proteins with a cysteine residue upstream of the CAAX motif (namely, H-Ras, N-Ras, and K-Ras4A), whereas the other CAAX-triggered events are irreversible (13–15). In lieu of a palmitoylation site, K-RasB possesses a cluster of lysine-rich sequences that may be responsible for increased affinity with the cell membrane, by electrostatically interacting with acidic phospholipids and other negatively charged groups on the inner membrane surface (11).

**RAS ACTIVATION**

Like other G proteins, Ras cycles between the GDP-bound inactive form and the GTP-bound active form. In the quiescent state, Ras exists in the GDP-bound form (16). After binding of an external ligand such as epidermal growth factor (EGF) to its receptor, dimerization of the receptor occurs, and the intrinsic receptor tyrosine kinase is activated. This is followed by auto-phosphorylation of specific tyrosine residues on the intracellular

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**caption**

Fig. 1. Pathway for the biosynthesis of isoprene molecules utilized in protein prenylation. The farnesyl group comprises 15 carbon chains. Two molecules of the 10-carbon geranyl group combine to form the 20-carbon geranylgeranyl group. Reactions blocked by the HMG CoA (i.e., 3-hydroxy-3-methylglutaryl-coenzyme A) inhibitors (such as lovastatin), phenylacetate, and the farnesyl transferase (FT) inhibitors are indicated. PP = pyrophosphate.

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**caption**

Fig. 2. Simplified scheme of the post-translational processing of H-ras. Farnesyl transferase (FT) transfers a farnesyl group (F) from famesyl-pyrophosphate (FPP) to the terminal cysteine of the CAAX motif (CVLS in the case of H-ras). The terminal tripeptide is cleaved by a specific endoprotease in the endoplasmic reticulum. The methyl donor for the reaction catalyzed by a prenyl protein-specific methyl transferase is S-adenosylmethionine. Palmitoylation of C-terminal cysteine residues occurs before membrane localization. CoA = coenzyme A.
portion of the receptor. These phosphorylated tyrosine residues then bind the sequence homology 2 (SH2) domains of adaptor proteins such as Grb2. These adaptor proteins contain not only an SH2 domain(s) but also an SH3 domain(s) that binds proline-rich motifs of other proteins, such as son of sevenless (SOS), a guanine dissociation stimulator (GDS) of Ras. The key adaptor molecule for Ras is Grb-2 that consists solely of one SH2 and two SH3 domains and links the activated EGF receptor to SOS. Such a complex formation recruits SOS, a cytosolic protein, into close proximity to Ras on the plasma membrane. The binding of SOS to Ras causes a change in the ras conformation and leads to the dissociation of GDP, which allows Ras to bind GTP and become active. Molecules such as SOS, which cause the dissociation of Ras from GDP and binding to GTP, are also called guanine nucleotide exchange factors (GNEFs) (Fig. 3). Ras must be in the plasma membrane not only for its SOS-mediated activation, but also for the ras-mediated activation of its downstream effectors. Activated ras activates several distinct effectors, such as the serine–threonine kinase rasaf-1, phosphoinositide 3'-kinase (PI3-K), and RalGDS. These downstream effectors activate several distinct signaling cascades, leading to either activation of certain genes, such as those encoding growth factors transforming growth factor-α and vascular endothelial growth factor (VEGF), or changes in actin cytoskeleton by activating Rho family G proteins (Fig. 4). Normally, these Ras-signaling cascades are only transiently activated because each normal Ras has low intrinsic guanine triphosphatase (GTPase) activity that gradually inactivates its own signaling function by hydrolyzing the bound GTP. More importantly, several distinct cytoplasmic GTPase-activating proteins (GAPs) stimulate the intrinsic GTPase activity of Ras, rapidly converting Ras from the active GTP form to the inactive GDP form. In this sense, the GAPs are attenuators of normal Ras. However, oncogenic mutations of ras not only reduce the intrinsic GTPase activity but also, more importantly, completely abolish the GAP-induced GTPase activation (17). Thus, unlike normal Ras, oncogenic Ras mutant proteins remain constitutively in the active GTP-bound form and continuously activate the downstream effectors. It is interesting that GAPs are bifunctional. For example, GAP1 acts not only as a normal Ras attenuator, but also as a Ras effector that mediates Ras-induced disruption of actin stress fibers through its N-terminal SH2 and SH3 domains and is required for Ras transformation (18).

The GTPase-activating protein NF1 is the product of the tumor suppressor gene, neurofibromatosis type 1 (NF1). Dysfunction of NF1 is associated with abnormal activation of normal Ras and causes neurofibromatosis type 1, which includes certain melanomas, clearly indicating that NF1 is important for the attenuation of normal Ras signaling. It is interesting that NF56, the minimal 56-amino-acid Ras-binding fragment of NF1, which no longer activates normal Ras GTPase, is still able to suppress malignant transformation caused by oncogenic Ras mutants, by blocking the interactions of Ras with all of its effectors (19). Fig. 3 illustrates how the GDP/GTP-bound Ras interacts with its activators GDSs (GNEFs) and attenuators/ effectors GAPs.

**RAS MUTATIONS IN HUMAN CANCERS**

This subject has been comprehensively reviewed (20). The role of ras genes in inducing malignant transformation is supported by several lines of evidence. First, oncogenic ras but not normal ras transfected into rodent fibroblasts renders them tumorigenic (21). Second, transgenic mice harboring oncogenic ras mutations have an increased incidence of tumor formation (22). Finally, a high frequency of ras mutations has been found in a variety of tumor types, both naturally occurring and experimentally induced. Identified mutations are limited to a very small number of sites (amino acids 12, 13, 59, and 61), all of which abolish GAP-induced GTP hydrolysis of the Ras proteins.

Such single-point mutations of the ras gene can lead to its constitutive activation of Ras protein. These mutated forms of Ras have impaired GTPase activity. Although they still bind GAP, there is no ‘off’ sign, since GTPase is no longer activated. This results in continuous stimulation of cellular proliferation. Mutations are frequently limited to only one of the ras genes, and frequency is dependent on tissue and tumor type (Table 1). Thus, ras gene mutations are rare in cancers of the breast, ovary, stomach, esophagus, and prostate; however, they are present in almost all adenocarcinomas of the pancreas and in 50% of colon and thyroid cancers. Mutations in colon and pancreatic cancers are found only in the K-ras gene. In cancers of the urinary tract and bladder, mutations are primarily in the H-ras gene; mutations are in the N-ras gene in leukemia. Thyroid carcinomas are unique in having mutations in all three ras genes (23–25). Overall, approximately 30% of all human neoplasms harbor a mutation in a ras gene. Mutations most frequently occur in K-ras and least often in H-ras. A critical experiment that underscored the importance of oncogenic ras in mammalian carcinogenesis was the demonstration that knocking out the activated ras gene in the human colon cancer cell lines DLD-1 and HCT-116 resulted in cell lines incapable of clone formation on soft agar and lacking tumorigenicity in nude mice. Thus, while human tumor cell lines may harbor multiple genetic mutations, deletion of an activated ras allele could suppress the expression of the malignant phenotype (26).

The ras oncogene has been reported to confer resistance to ionizing radiation (27,28). However, there have been conflicting results (29,30). An explanation may be the genetic heterogeneity
of the various cell lines studied and the possible mutation of other proto-oncogenes, such as raf and myc, that may interact with ionizing radiation (28).

RAS MUTATIONS AND PROGNOSIS

Numerous studies have been performed evaluating the prognostic importance of oncogenic ras mutations in human tumors. These studies have focused predominantly on hematologic malignancies (leukemia and multiple myeloma) and pancreatic, non-small-cell lung, and colorectal cancers. Results to date have been conflicting (31,32). In colon cancer, some studies (33,34) failed to show any correlation between K-ras mutation and patient outcome. However, other studies (35), including the largest study to date with 1413 individuals (36), demonstrated that the presence of K-ras mutations is a poor prognostic indicator. Likewise, in non-small-cell lung cancer, while some studies are negative or equivocal (37,38), the preponderance of data favors ras mutations as being a negative prognostic factor (39–42).

Table 1. ras mutations in human tumors

<table>
<thead>
<tr>
<th>Cancer or site or tumor</th>
<th>Mutation frequency, %</th>
<th>Predominant ras isoform*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-small-cell lung cancer (adenocarcinoma)</td>
<td>33</td>
<td>K</td>
</tr>
<tr>
<td>Colorectal</td>
<td>44</td>
<td>K</td>
</tr>
<tr>
<td>Pancreas</td>
<td>90</td>
<td>K</td>
</tr>
<tr>
<td>Thyroid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular</td>
<td>53</td>
<td>H, K, N</td>
</tr>
<tr>
<td>Undifferentiated papillary</td>
<td>60</td>
<td>H, K, N</td>
</tr>
<tr>
<td>Papillary</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Seminoma</td>
<td>43</td>
<td>K, N</td>
</tr>
<tr>
<td>Melanoma</td>
<td>13</td>
<td>N</td>
</tr>
<tr>
<td>Bladder</td>
<td>10</td>
<td>H</td>
</tr>
<tr>
<td>Liver</td>
<td>30</td>
<td>N</td>
</tr>
<tr>
<td>Kidney</td>
<td>10</td>
<td>H</td>
</tr>
<tr>
<td>Myelodysplastic syndrome</td>
<td>40</td>
<td>N, K</td>
</tr>
<tr>
<td>Acute myelogenous leukemia</td>
<td>30</td>
<td>N</td>
</tr>
</tbody>
</table>

*K = Kirsten; H = Harvey; N = neuroblastoma.

Possible explanations for the inconsistent results include the predominantly retrospective nature of these studies, the small numbers of patients studied, and the inability to correct for other confounding prognostic factors.

CYTOSOLIC TARGETS OF RAS

Activated ras utilizes several downstream effectors, the most characterized of which is Raf-1 (43). Other effectors include Rac, Rho, and PI3-K.

Raf-1

Mitogen-activated protein (MAP) kinase cascades lie in a three-kinase-signaling module involved in transmitting membrane signals to the cell nucleus. An MAPK module consists of MAP kinase or extracellular signal-regulated kinase (ERK) activated by an MAP/ERK kinase (MEK or MAPKK) which, in turn, is activated by an MEK kinase (MEKK or MAPKKK). One such MEKK, which is the most well-characterized downstream effector of Ras, is the serine–threonine kinase Raf-1. This protein is recruited by Ras-GTP to the plasma membrane, where Raf is activated by an as yet unknown factor (44–52). Localization of Raf to the plasma membrane is essential for its activation. Thus, fusion of Raf to the C-terminal membrane-localization signal of K-ras leads to its constitutive localization to the plasma membrane and bypasses the need for Ras (44,45). Once activated, Raf phosphorylates MEK, which, in turn, phosphorylates ERK (51). MAPK activation results in phosphorylation and activation of ribosomal S6 kinase and transcription factors, such as c-Jun, c-Myc, and c-Fos, resulting in the switching on of a number of genes associated with proliferation (43) (Fig. 4). The critical effector function of Raf is supported by several studies. First, dominant-negative mutants of Raf can impair Ras-transforming activity (53,54). Second, constitutively activated forms of Raf possess transforming activity comparable to that of Ras (55,56) and are themselves sufficient to transform some
murine cells (57–59). However, Raf is clearly not the sole effector of Ras, since Ras/Raf-independent pathways for MAP kinase activation exist (43).

**Rac and Rho**

The G proteins Rac and Rho cycle between GDP- and GTP-bound forms and are regulated by factors analogous to GNEFs and GAPs (16) (Fig. 3). These two proteins have been shown to be activated by Ras-GTP (60,61). Through their regulation of the active cytoskeleton, Rac and Rho are critical in cellular processes, such as formation of focal adhesions, filopodia, stress fibers, and membrane ruffling (48). All of these processes that can be activated by oncogenic ras are important for the invasive phenotype of transformed cells (62,63).

**Phosphoinositide 3’-Kinase**

Another ras effector is PI3-K, a complex of two distinct subunits, the catalytic subunit of 110 kd (p110) and the regulatory subunit of 85 kd (p85). Ras-GTP binds the catalytic p110. This interaction leads to a modest increase in PI3-K activity, increasing the concentration of 3’-phosphorylated inositol lipids (64). One of the PI3-K products, phosphatidyl inositol 3,4,5-triphosphate (PIP3), activates Rac (65). Rac, in turn, induces the production of phosphatidyl 4,5-biphosphate (PIP2) by activating PIP4/PIP5 kinases, leading to uncapping of actin filaments at the plus-end (66) and eventually induces membrane ruffling (67). Both PI3-K and Rac are required for ras transformation. Thus, the drugs SCH51344 and cytochalasin K that block Rac-induced membrane ruffling or the PIP2-sequestering SH3 protein HS1 reverse ras transformation (68–70). Cellular processes of fundamental importance in oncogenic transformation, such as prevention of apoptosis, increase in cell motility, and invasiveness, have been linked to PI3-K signaling (71–76). In addition, the suppression of c-Myc-induced apoptosis by Ras may be mediated through PI3-K (77,78). Among the many potential downstream effectors of PI3-K, only the serine–threonine kinase Akt (protein kinase B) has been clearly identified (79). The downstream targets of Akt can be broadly classified into survival and death factors, such as the pro-apoptotic proteins Bad (80) and caspase 9 (81), whose expression is decreased by Akt, and the kinase mammalian target of rapamycin (mTOR). The downstream targets of mTOR, p70S6 kinase and PHAS-1, are important in cell cycle progression (82,83) (Fig. 4).

**Serine–Threonine Kinase MEKK1**

The serine–threonine kinase MEKK1 is implicated in the stress-response pathway, whose downstream targets include the MAP kinases c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK) (Fig. 4) (84,85). The MEKK1/JNK/SAPK cascade has been shown to induce apoptosis under certain circumstances (54). MEKK1 is activated by Ras-GTP through the Raf/Cdc42 pathway (86) (Fig. 4). Even though the primary target of MEKK1 appears to be JNK, recent evidence suggests that MEKK1 can activate the MEK/ERK pathway independent of Raf-1 (Fig. 4). JNK may be the target of Ras in an alternate pathway involving Rac/Rho/CDC42 and not the MAP kinase pathway. Cross-talk, however, almost certainly exists between these two pathways (87).

**Other Potential Cytosolic Ras Effectors**

Other Ras effectors include protein kinase C-zeta, phospholipase C (88), and RalGDS (50–52). RalGDS is a guanine nucleotide dissociation stimulator specific for Ral, which is recruited to the plasma membrane by activated ras. However, the cellular role of Ral family proteins (RalA and RalB) is unclear. Similarly, the nature and function of Ras signaling through protein kinase C and phospholipase C remain unclear. Clearly, multiple Ras targets cooperate to produce the plethora of effects that occur after Ras activation.

**Nuclear Targets of Ras**

The critical nuclear target of the Ras/Raf/MEK/MAP kinase pathway is the transcription factor Fos. The Fos protein forms a heterodimer with jun to yield the active AP1 complex. The Jun protein is activated through phosphorylation of its amino terminal serine residues at positions 63 and 73 by JNK. Fos can also be phosphorylated by JNK. But it appears that MAP kinase is unable to phosphorylate Jun (89). The JNK/JUN pathway, which parallels the Raf/MEK/MAP kinase pathway, is called the stress-activated-protein (SAP) pathway (Fig. 4).

**Other Ras Proteins**

**R-ras**

The small GTPase R-ras displays a less potent transforming activity than the oncogenic ras products (N-, K-, and H-ras). Although R-ras can interact with Raf-1 and RalGDS, these interactions are weak, as is the activation of the downstream Raf/Mek pathway in intact cells (90). R-ras is approximately 55% identical to the Ras proteins. Unlike mutated Ras proteins, R-ras mutants do not induce morphologic transformation in NIH 3T3 cells. However, NIH 3T3 transfectants of R-ras form colonies in soft agar, which form tumors in nude mice. Thus, constitutively activated R-ras can promote malignant transformation, but its mechanism of action may be distinct from that of the other Ras proteins. It has been shown that, like the Ras proteins, R-ras activates the PI3-K/Akt pathway but differs in not activating the Raf/MEK pathway. The exact cellular location of R-ras is unknown; however, since Ras recruits Raf to the plasma membrane before activation, R-ras may have an intracellular location. R-ras has been reported to also promote integrin activation, presumably through a PI3-K pathway (91).

**M-ras**

M-ras shares approximately 55% sequence identity with K-ras and TC21, a ras-related family member (92). Similar to K- and H-ras, expression of mutationally activated M-ras in NIH 3T3 cells results in cellular transformation. M-ras only weakly activates the MAP kinase pathway. In a yeast two-hybrid system, M-ras interacted poorly with multiple Ras effectors, such as Raf, RalGDS, and PI3-K. These data indicate that M-ras may signal through multiple alternate pathways. Transfection experiments of M-ras complementary DNA into fibroblasts suggest that this protein plays a role in reorganization of the actin cytoskeleton (93).

**Function of Normal Ras Proteins**

**Cell Proliferation and Differentiation**

The precise functions of Ras proteins continue to be elucidated. As described above, Ras proteins are activated in response to an array of extracellular stimuli that influence growth and differentiation. These stimuli include a wide variety of growth...
factors, such as EGF, platelet-derived growth factor, interleukin 2, interleukin 3, and granulocyte–macrophage colony-stimulating factor. The cell surface receptors for these mitogenic molecules are typically receptor tyrosine kinases or receptors with associated nonreceptor tyrosine kinases. Thus, the Ras proteins appear to be essential components of tyrosine kinase-mediated mitogenic signaling pathways. Under normal conditions, quiescent cells in G1 generally have less than 5% of their total Ras pool in the active state compared with nearly 50% upon mitogenic stimulation (94).

Ras-dependent signaling, however, may mediate differentiation or proliferation, depending on the cell type and transmembrane receptor (95). For example, activated Ras has mitogenic effects in fibroblasts, while it induces growth arrest and extension of neurite-like processes in the rat PC12 pheochromocytoma cell line (96).

Direct interactions between Ras and JNK and jun have been described. When UV radiation-activated 3T3/4A cells are immunoprecipitated with antibodies, Ras, JNK, and jun colocalize (97). This evidence as well as other evidence reviewed by Pinkus et al. (98) suggests that oncogenic Ras may interact directly with JNK/Jun, bypassing the normal control mechanisms that prevail for the activated normal protein. The normal protein activates the Raf/MEK/MAP kinase pathway and cyclin. The Rac/Rho/CDC42 pathway is also stimulated, which activates JNK in a regulated stepwise manner (Fig. 4).

Cell Cycle Regulation

Ras-mediated activation of the MEK/MAP kinase pathway increases cellular levels of cyclin D1, but other effector pathways may also be important in cyclin D1 induction. Increased levels of cyclin D1 promote the progression of cells through the G1 checkpoint and into S phase, leading to proliferation. Ras-mediated activation of the PI3-K and RalGDS pathways also leads to G1 progression. Gille and Downward (99) demonstrated that PI3-K activity is required for the expression of endogenous cyclin D1 and for S-phase entry after serum stimulation of quiescent NIH 3T3 fibroblasts. This activated PI3-K-induced cyclin D1 transcription and E2F activity are mediated, at least in part, by the serine–threonine kinase Akt/PKB and, to a lesser extent, the Rho family GTPase Rac. In addition, both activated RalGDS-like factor and Raf stimulate cyclin D1 transcription and E2F activity and act in synergy with PI3-K (99). Thus, multiple Ras-effector pathways mediate the effects of ras on progression through the cell cycle.

Integrins Signaling/Cell Survival

Integrins are a family of adhesion receptors for extracellular matrix proteins. Integrins also generate signals that influence a variety of cellular functions, including migration, proliferation, and apoptosis. Many signaling pathways downstream of integrins have been identified and characterized. In particular, integrins regulate focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase, and result in the transient activation of MAP kinases (100).

Accumulated data indicate that the integrin-signaling pathway may share common elements with the Ras signal transduction cascade. It has been demonstrated that the Raf/MEK/MAPK pathway is activated subsequent to integrin-mediated adhesion of mouse NIH 3T3 fibroblasts. Evidence also suggests that MAP kinase is downstream of MEK in the integrin-signaling pathway. However, conflicts exist in the data. Chen et al. (101) demonstrated that, in contrast to the receptor tyrosine kinase-signaling cascade, integrin-mediated signal transduction appears to be independent of Ras. Dominant negative inhibitors of Ras-dependent signaling failed to block integrin-mediated activation of MEK. In addition, while treatment with EGF increases Ras-GTP, little effect was observed in response to integrin-dependent cell adhesion. On the other hand, Clark and Hynes (102) showed that inhibition of Ras signaling by expression of another dominant-inhibitory mutant of Ras (N17Ras) in NIH 3T3 cells blocked adhesion-dependent activation of ERK2, although FAK was still activated in these cells. Furthermore, activation of this Ras/MAP kinase pathway activated cytosolic phospholipase A2, leading to the release of arachidonic acid metabolites, and N17Ras also inhibited these events. However, N17Ras expression does not inhibit cell adhesion, spreading, or focal contact and stress fiber formation (102). Taken together, these results and other findings (103) suggest that, while integrin-dependent activation of the MAP kinase/ERK pathway may be Ras dependent, the integrin-dependent activation of FAK and several morphologic events are Ras independent. Thus, integrin-mediated signals involved in regulating cell morphology appear to diverge from those regulating MAP kinase activation at a level upstream of Ras activation. It is interesting that the Ras-related GTPase, R-ras, modulates integrin affinity, inducing an increase in cellular adhesion and migration on collagen but not on fibronectin, suggesting that R-ras signals to specific integrins (104). It has been shown that R-ras induces migration on collagen through a combination of PI3-K and protein kinase C, but not MAP kinase activation, which is distinct from the other Ras family members, Rac, Cdc42, and N- and K-ras. Thus, R-ras communicates with specific integrin-α cytoplasmic domains through a unique combination of signaling pathways to promote cell migration and invasion.

Angiogenesis

Oncogenic ras mutations have been implicated in tumor metastasis and angiogenesis. Data suggest that this is a direct effect of the Ras oncoprotein and not simply an indirect consequence of the proliferative properties of Ras. Transformation of cells by oncogenic ras mutants has been shown to increase the expression of key metalloproteinases, such as gelatinase and stromelysin, which are involved in tumor metastasis (105). Moreover, the expression of thrombospondin, an extracellular matrix molecule that is decreased in metastatic tumors, is also reduced on transfection of cells with viral K-ras (106). The expression of the angiogenic growth factor VEGF is increased in K-ras-transformed and H-ras-transformed epithelial cells through the Raf pathway. Furthermore, genetic disruption of the mutant K-ras allele in human cancer cells was associated with a reduction in VEGF activity (107).

Therapeutic Implications

Because of the high percentage of human tumors harboring oncogenic ras mutants, interrupting the Ras-signaling pathway has been a major focus of new-drug-development efforts. The major approaches taken are as follows: 1) the inhibition of Ras protein expression through ribozymes, antisense oligonucleotides, or RNAs; 2) the prevention of membrane localization of Ras; and 3) the inhibition of downstream effectors of Ras function.
Inhibition of Ras Protein Expression: Antisense Oligonucleotides

The antisense approach involves targeting specific RNA sequences to block translation of the RNA message into protein. Oligonucleotides, which are complementary to messenger RNA (mRNA) transcripts of the activated ras oncogene, have been utilized to decrease Ras protein expression. These oligonucleotides hybridize to complementary mRNA sequences and decrease protein expression through a variety of mechanisms, including RNase H-mediated cleavage of hybridized ras mRNA. K-ras antisense approaches have utilized large constructs incorporated into plasmids or viral vectors. Roth and co-workers (108,109) incorporated a 2-kilobase K-ras antisense sequence into an adenoviral vector. This vector generated K-ras antisense RNA and reduced K-ras protein levels, inhibiting the growth of the H460A NSCLC cell line in culture (108). This K-ras viral construct was successfully administered intratracheally to nude mice bearing implanted human lung cancers. Statistically significant activity was observed, with 87% of treated mice being tumor free compared with 10% of control mice (108,109). A different K-ras antisense RNA, generated through liposomal transfection of a plasmid construct, has demonstrated inhibition of human pancreatic cancer cells bearing the K-ras mutation in vitro. No activity was observed in cell lines with wild-type Ras. These plasmids were effective in vivo, when they were injected intraperitoneally into rodents with pancreatic cancer xenografts injected into the peritoneum (110). A persistent problem with the K-ras antisense strategies had been the inability to deliver intact antisense RNA agents into tumor cells. As described above, gene therapy approaches of direct intratumoral injection or regional delivery have been utilized. In recent years, the distribution of phosphorothioate oligodeoxynucleotides, presumably by endocytosis in rodent tissues after intravenous administration, has been demonstrated (111). This allows for systemic administration of antisense oligonucleotides. An investigational phosphorothioate antisense oligodeoxynucleotide, ISIS 2503, which was designed to hybridize to the 5′-untranslated region of human H-ras mRNA, has entered clinical trials. Since this construct does not selectively target the point mutations in codon 12, 13, or 61 of the H-ras gene, there is a broad anti-H-ras effect. It is interesting that, in preclinical studies, antitumor effects were independent of ras gene status. Activity was observed in MiaPACA2 pancreatic cancer cells bearing K-ras mutations and in HT-29 colon cancer cells that possess wild-type ras. Phase I studies have been completed. The dose and schedule recommended for further studies are 6 mg/kg administered as a continuous 14-day infusion, repeated every 21 days. Interim results indicate a tolerable toxicity profile, with moderate thrombocytopenia and fatigue as the only adverse events. An indication of biologic activity has been seen, with mild tumor shrinkage in a patient with sarcoma (112). Phase II studies are ongoing, and a phase I combination study with gemcitabine has been completed (113).

Inhibition of Ras Membrane Localization

Farnesyl protein transferase and its inhibitors. Mammalian FT is a zinc metalloenzyme, which exists as a heterodimer (114). The 48-kd α-subunit is identical to that of GGT-I. The 45-kd β-subunit appears to contain the binding sites for both substrates (peptide and isoprenoid). The CAAX motif is crucial in recognizing peptide substrates. The Α1 position has a relaxed amino acid specificity with basic and aromatic side chains tolerated, whereas the Α2 and X positions have strict specificity criteria (115,116). For example, the substitution of an aromatic side chain at Α2 creates a molecule, which is a competitive inhibitor of FT (117). Either substrate is able to bind independently to FT, but the preferred sequence is isoprenoid binding followed by peptide binding. Product release after these processes appears to be the rate-limiting step (118). In addition to zinc, FT requires Mg2+ for activity. However, the exact role of zinc and magnesium ions in catalysis of the transferase reaction remains unknown at present.

Known substrates for FT in mammalian cells include the Ras proteins, the nuclear lamin proteins lamin B (119) and prelamin A (120), cyclic guanosine monophosphate phosphodiesterase α (121), rhodopsin kinase (122), a peroxisomal protein PxF of unknown function (123), and the γ-subunit of the retinal protein transducin (124) (Table 2).

Initial approaches to FT inhibition involved the use of general inhibitors of isoprenylation. Synthesis of farnesyl groups can be blocked by the HMG CoA (i.e., 3-hydroxy-3-methylglutaryl-coenzyme A) reductase inhibitors, such as lovastatin, and the mevalonate pyrophosphate decarboxylase inhibitor phenylacetate (Fig. 1). These agents have been tested in early clinical trials. While antiproliferative activity was seen in a range of tumors in vitro, phase I trials suggested activity only in glioma (125). However, because of the nonspecific nature of the inhibition of these agents (lovastatin is 100-fold more potent an inhibitor of cholesterol biosynthesis), toxicity was substantial in subsequent studies.

Specific inhibitors of FT hold greater promise as anticancer agents. The historic development and structural diversity of FT inhibitors (FTIs) have been reviewed (126,127). One of the first true inhibitors of FT was the tetrapeptide CVF (i.e., cysteine–valine–phenylalanine–methionine), which competed with the CAAX motif. While this agent was not clinically viable because of its inability to enter cells, it served as a prototype for the development of other therapeutic candidates. Subsequent developments focused on peptide CAAX inhibitors, which were modified to enter cells.

The FTIs fall into three main classes: 1) the CAAX competitive inhibitors, such as L731735, L744832, SCH66336, and R115777 that compete with the CAAX portion of ras for FT; 2) the FPP competitive inhibitors, such as PD 169451 and RPR

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-, K-, and N-ras</td>
<td>Growth; differentiation;</td>
<td>(6–9,12–14)</td>
</tr>
<tr>
<td>Rho-B and Rho-E</td>
<td>inhibits apoptosis</td>
<td>(61,151,152)</td>
</tr>
<tr>
<td>HDJ-2</td>
<td>Cell cycle control; cytoskeletal organization</td>
<td>(126,137,174)</td>
</tr>
<tr>
<td>Lamins A and B</td>
<td>Chaperone protein</td>
<td>(119,120)</td>
</tr>
<tr>
<td>PxF</td>
<td>Nuclear membrane structure</td>
<td>(123)</td>
</tr>
<tr>
<td>Transducin</td>
<td>Peroxisomal protein of unknown function</td>
<td></td>
</tr>
<tr>
<td>Rhodopsin kinase</td>
<td>Visual protein</td>
<td>(124)</td>
</tr>
<tr>
<td>Cyclic guanosine monophosphate phosphodiesterase α</td>
<td>Visual protein</td>
<td>(122)</td>
</tr>
<tr>
<td>Phosphorylase kinase α and β</td>
<td>Visual signal transduction</td>
<td>(121)</td>
</tr>
</tbody>
</table>

Table 2. Partial listing of farnesylated mammalian proteins

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130401 that compete with the substrate FPP for FT; and 3) the bisubstrate inhibitors, such as BMS-186511 (128-132). Currently, four FTIs are in clinical trials worldwide, with several more at different levels of preclinical development. Two of these, R115777 and SCH66336, are orally active heterocyclic compounds and are in phase II studies. The other two agents, L778,123 and BMS-214662, are administered intravenously and are in phase I trials. The clinical safety and activity of R115777, SCH66336, and L778,123 have been recently reported. Neutropenia was dose limiting for R115777, when it was administered twice daily for 21 days at doses of 400 and 500 mg (133,134). In an earlier study (131) in which the drug was administered for 5 days every 21 days, myelosuppression was not seen. Moderate fatigue was the major toxicity. Similarly, myelosuppression was rare in a study of SCH6636, where the agent was given twice daily for 7 days, every 21 days. Diarrhea was dose limiting. Fatigue was also observed (135). Some incidence of myelosuppression was seen when this agent was administered on a continuous daily schedule (136). L778,123 was administered as a continuous 7-day infusion. Cardiac toxicity manifest as Q-T prolongation was reported (137). Some of these toxic effects, such as myelosuppression and fatigue, may represent consequences of FT inhibition. Myelosuppression may represent the inhibition of the farnesylation of a protein important in cytokine-mediated signaling required for bone marrow progenitor cell development (138). Fatigue may represent the inhibition of farnesylation of phosphorylase kinase α and β, which are involved in skeletal muscle function (126). The unique toxic effects for each agent may be structurally related.

Because of cardiac conduction abnormalities (which have not been seen with the other agents), the clinical development of L778,123 has been discontinued. In addition to phase I studies, preliminary results of a phase II study of R115777 in patients with metastatic breast cancer previously receiving one hormonal or chemotherapy treatment regimen have been reported. Twenty-seven evaluable patients received a continuous oral dose of 300 mg twice daily. The first six patients received a dose of 400 mg, which was later reduced to 300 mg because severe neutropenia occurred by day 26. Confirmed partial responses in soft-tissue disease were seen in 12% of patients. Another 35% of patients had stable disease for at least 3 months (139).

The first preliminary report of a study of these agents in hematologic malignancies has been published (140). R115777 was tested in patients with refractory leukemia. Neutropenia was the major toxicity seen in that study. The recommended phase II dose was 600 mg given twice daily for 21 days. A 30% response rate was reported.

Combination studies of FTIs and several standard cytotoxic agents are ongoing. However, only three full reports of preclinical cytotoxic synergy between FTIs and standard chemotherapy agents have been published. The first study (141) reported on combinations of the FTI L744832 with paclitaxel or epothilone in cultured cells. The second study (142) evaluated SCH66336 with vincristine, cyclophosphamide, or 5-fluorouracil in transgenic mice. The third study (143) evaluated FTI2148 with paclitaxel, gemcitabine, or cisplatin in nude mice. Of these FTIs, only SCH66336 is undergoing clinical testing.

Phase I combination studies of R115777 and SCH66336 with gemcitabine have been reported in preliminary form. Initial results (144,145) suggested that the R115777 and gemcitabine combination may be better tolerated. Preliminary evidence of activity was seen in both studies. A combination study of SCH66336 with paclitaxel (146) yielded impressive preliminary results with partial responses in six of 18 evaluable patients, including patients whose disease had previously progressed while on taxanes.

Currently, at least one FTI, R115777, is undergoing phase III clinical testing in pancreatic and colorectal cancers.

While the FTIs clearly do not or only partly target Ras, these agents appear to have clinical activity in leukemia and in some solid tumors. Inhibition of FT by these compounds in normal patient tissues and tumor cells has been documented (135,147). Their toxic effects appear to be manageable. Ongoing phase II studies will determine their extent of clinical activity. From initial results, however, it may be likely that their single-agent activity may not compare with that of standard cytotoxic agents and that they may have to be used entirely in combination, both with cytotoxic chemotherapy and with radiation therapy.

**Mechanism of action.** Although FTIs clearly inhibit Ras farnesylation, it is unclear whether the antiproliferative effects of these compounds result exclusively from effects on Ras. In vitro studies suggest that both N-ras and K-ras are low-affinity substrates for GGT-I. Geranylgeranylation of K-ras and N-ras proteins has been observed in intact cells treated with FTIs (148). Furthermore, geranylgeranylated forms of Ras have oncogenic transformation potential when they are overexpressed. Despite this alternative prenylation pathway, FTIs demonstrate in vitro and in vivo antitumor activity in cells with activated K-ras mutations. In addition, several cell types with no ras mutations are sensitive to FTIs, both in vitro and in vivo. Collectively, these findings argue that inhibition of the farnesylation of other proteins might also contribute to the observed antitumor properties of FTIs (149,150). At least two hypotheses have been advanced to explain the mechanism(s) of cytotoxicity of the FTIs:

First, RhoB, a 21-kd G protein that regulates receptor trafficking, has been implicated as the prenylated target of FTIs. Like K-rasB and N-ras, RhoB can be either farnesylated or geranylgeranylated. The different prenylated forms of RhoB appear to have opposing cellular effects. Farnesylated RhoB promotes cellular transformation, whereas geranylgeranylated RhoB suppresses the transformed phenotype, even in cells transfected with mutant genes. According to the Rho hypothesis, the antitumor effects of FTIs depend on the accumulation of geranylgeranylated RhoB (151). There are elegant data supporting this hypothesis (152). However, the hypothesis fails to account for the antitumor effects of GGT-I or dual FT and GGT-I inhibitors (153). In addition, Chen et al. (154) have demonstrated that both farnesylated and geranylgeranylated RhoB inhibit anchorage-dependent and anchorage-independent growth and suppress tumor growth in nude mice.

A second hypothesis has been advanced recently by Jiang et al. (155), who have demonstrated that FTIs inhibit PI3-K/AKT-mediated growth factor- and adhesion-dependent survival pathways and induce apoptosis in human cancer cells that overexpress AKT. It is interesting that overexpression of AKT2 but not of oncogenic H-ras sensitized NIH 3T3 cells to FTIs. Integrin-dependent activation of AKT2 was also blocked by FTIs (155). These data and other results (156) suggest that FT inhibition of human tumor growth may be mediated through inhibition of a farnesylated protein associated with the PI3-K/AKT2-mediated cell survival pathway. Consistent with this hypothesis is a report (157) indicating that FTIs block activation of p70s6k, which is
downstream of PI3-K. As a consequence of the alternative prenylation pathway for N-ras and K-ras, it has been shown in ras-transformed cells that the farnesylation of K- and N-ras is more resistant to FTIs than the farnesylation of H-ras (153,158,159). In particular, inhibition of the prenylation of K-ras in human tumor cell lines was found to be resistant to FTIs and required both FTIs and GGT-I inhibitors (160).

These findings may have implications for the utility of FTIs as single agents in tumors with a predominance of K-ras mutations, such as non-small-cell lung, pancreatic, and colon carcinomas. Conversely, these agents may show activity in tumors with H-ras mutations and in tumors with a highly activated wild-type Ras pathway because of overexpression of growth factor receptors, such as breast and ovarian cancers.

Geranylgeranyl transferase type 1 inhibitors. Interest in inhibitors of GGT-I has increased recently, with recognition that K-ras 4B, the most frequently mutated form of ras in human cancers, can be geranylgeranylated (161). Using a peptidomimetic approach, Sebti and colleagues have pioneered the search for GGT-I inhibitors (162,163) and have reported on a series of potent, highly selective inhibitors of GGT-I (164). No GGT-I inhibitors are currently in clinical trials. However, it has been shown that GGT-I inhibitors can affect critical cellular processes. These include inhibition of platelet-derived growth factor receptor tyrosine kinase phosphorylation (165) and growth arrest of human neoplastic cells in G1, presumably through inhibition of RhoA geranylgeranylation (166). While FTIs block or reverse malignant growth in a wide variety of human tumor cell lines, they appear to have little effect on normal cell proliferation, viability, or differentiation. This selectivity suggests that these agents identify a unique feature of neoplastic cell signaling and may explain the relative lack of toxicity seen in current clinical trials. In contrast to the FTIs, GGT-1 inhibitors seem to have substantial effects on cell signaling in normal cells, inducing cell cycle arrest and apoptosis (168). At this time, the impact of such findings on the clinical development of GGT-I inhibitors is unclear.

**Interruption of Signaling Pathways Downstream of Ras**

**Raf kinase inhibitors.** As described above, c-Raf kinase acts downstream of Ras in the MAP kinase pathway. Evidence (169) also suggests that c-Raf kinase may also be activated by other mechanisms such as bcl-2. ISIS 5132 is a 20-mer phosphothioate antisense oligonucleotide, which inhibits c-Raf kinase. Initial proof-of-principle studies demonstrated antiproliferative effects in cultured human cell lines with concomitant reduction in c-raf kinase mRNA (170). Phase I testing of different infusion schedules have been completed. Toxic effects were mild fever and evidence of complement activation, which is clinically insignificant (171). Phase II studies are under way in prostate, colorectal, and ovarian cancers. In contrast to large molecules, such as oligonucleotides and peptides or proteins (vaccines and antibodies), chemicals of relatively low molecular weight (small molecules), which inhibit c-Raf kinase, have been described. IC50 (i.e., drug concentrations that inhibit the enzyme by 50%) values for the isolated enzyme are in the 2–50-nM range, and the proliferation of several human cell lines was potently inhibited in vivo and in vitro (172). Phase I clinical studies are expected to commence this year with some of these molecules.

**MEK inhibitors.** As described previously, sequential activation of MAPK kinase (MAPKK or MEK1) and MAP kinase (ERK1/2) occurs downstream of Ras. MAP kinase, in turn, phosphorylates downstream substrates involved in cellular responses, such as cytoskeletal changes and gene transcription. Conditional MAP kinase activation is important in gene regulation, promoting G1 cell cycle progression before DNA replication and spindle assembly during both meiotic and mitotic cell division, among other processes. Inappropriate activation of the MAP kinase pathway, through mutations introduced via oncogenes, is a feature of many neoplasms. Molecules, such as MEK, are, therefore, potential targets for cancer therapy. Sebolt-Leopold et al. (173) have reported the discovery of PD184322, a highly potent and selective inhibitor of the upstream kinase MEK, that is orally active. Tumor growth was inhibited by as much as 80% in mice implanted with col 26 and HT 29 colon carcinomas after treatment with this inhibitor. Efficacy was achieved with a wide range of doses with no signs of toxicity and correlated with a reduction in the levels of activated MAP kinase in excised tumors. These data indicate that MEK inhibitors represent a promising, nontoxic approach to the interruption of the Ras/MAP kinase pathway for cancer therapy (173).

It has been argued that blocking a downstream target such as MEK may abrogate a number of different signaling pathways, which impinge on this intermediary molecule. Such blockage may allow for targeting of a broad range of tumors but increases the potential of toxic effects. On the other hand, blocking an upstream target such as EGF receptor (HER-1) may limit the range of tumors that can be treated to only those that aberrantly express this receptor. This selectivity may, however, reduce the potential for toxicity. At this time, it is unclear if either of the two approaches will be superior. Ongoing clinical trials will resolve this issue.

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Notes

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