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The *CAAX* Peptidomimetic Compound B581 Specifically Blocks Farnesylated, but Not Geranylgeranylated or Myristylated, Oncogenic Ras Signaling and Transformation*

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Recently developed *CAAX* peptidomimetic compounds have been shown to be potent and specific inhibitors of farnesyl protein transferase activity and to block the growth of Ras-transformed cells. However, whether this growth inhibitory action is specifically a consequence of blocking oncogenic Ras signaling has not been determined. To address this question, we have utilized mutants of the normally farnesylated oncogenic Ras protein (Ras-F) that are modified by alternative lipids, a geranylgeranyl isoprenoid (Ras-GG) or the fatty acid myristate (Myr-Ras), to determine the specificity of the *CAAX* peptidomimetic compound, B581. Like Ras-F, both Ras-GG and Myr-Ras are membrane-associated and transforming. Unexpectedly, NIH 3T3 cells transformed by each of the three Ras mutants underwent morphological alteration to a less transformed, but not normal, morphology. However, B581 inhibited the ability of only Ras-F-transformed cells, but not Ras-GG- or Myr-Ras- (or Raf-) transformed cells, to grow in soft agar. Furthermore, although all three lipid-modified versions of Ras stimulated mitogen-activated protein kinase activation, and both Jun and Elk-1 transcriptional activity, B581 inhibited only farnesylated Ras activation of these three downstream components of Ras signaling. Therefore, B581 prevents the growth of Ras-transformed cells by specifically antagonizing Ras-mediated signaling.

Ras proteins function as critical GDP/GTP-regulated relay switches that control signaling from upstream receptor tyrosine kinases downstream to a cascade of serine/threonine kinases that include the mitogen-activated protein kinases (MAPKs[1]; also known as extracellular signal-regulated kinases or ERKs) (1, 2). This cascade is triggered by Ras interaction with the Raf-1 proto-oncogene protein, which in turn activates the MAPK/ERK kinases (MEKs) (3). MEKs are dual specificity kinases that activate MAPKs, which translocate to the nucleus and phosphorylate transcription factors to alter gene expression (4). Oncogenic Ras triggers constitutive activation of this cascade of serine/threonine kinases, which results in deregulated gene expression and transformation (5).

Ras proteins mature by undergoing a series of posttranslational modifications (farnesylation, proteolysis, and methylation) triggered by a consensus COOH-terminal *CAAX* (C, cysteine; *A*, aliphatic, *X*, S, M, or C) tetrapeptide sequence (6). The attachment of the isoprenoid farnesol to the cysteine residue of the *CAAX* motif, which is recognized by the Ras protein: farnesyltransferase (FPTase) enzyme, is the critical modification required for Ras membrane association and transforming activity (6). Nonfarnesylated mutants of oncogenic Ras are cytosolic and completely defective for transformation. However, both Ras membrane association and transformation can be restored either by *CAAX*-signaled geranylgeranylation (7, 8) or by addition of an NH_2-terminal myristylation signal sequence (9, 10). Thus, while it is clear that membrane association is essential for Ras transformation, the precise role of farnesylation remains unclear.

The importance of farnesylation to Ras transforming function has prompted the development of FPTase inhibitors that may serve as selective agents to block oncogenic Ras function in human tumors (11). In particular, the generation of compounds that are based on the structure of the Ras *CAAX* tetrapeptide sequence has resulted in several potent and specific inhibitors of FPTase activity (11). Although a second enzyme involved in protein prenylation, geranylgeranyl protein transferase I, recognizes a related *CAAX* tetrapeptide signal (where *X* is L), these FPTase-directed *CAAX*-based compounds show strong and preferential inhibition of FPTase activity *in vitro* and *in vivo* (12–15).

Present evidence suggests that *CAAX* peptidomimetics can selectively block Ras transforming activity. For example, the inhibition of colony formation in soft agar of Ras-transformed, but not Mos- or Raf-transformed, cells suggests that these compounds may be specific inhibitors of oncogenic Ras function *in vivo* (12, 13). However, whether this inhibitory action is a direct consequence of inhibiting oncogenic Ras signaling is not known.

To address this question, we have utilized mutants of oncogenic Ras that are modified by alternative lipids. Since both geranylgeranylation and myristylation can restore membrane association and transforming activity to nonfarnesylated Ras, cells transformed by these lipid variants provide ideal controls to evaluate the specificity and mechanism of action of *CAAX* peptidomimetics. In this study, we show that the B581 *CAAX* peptidomimetic compound (14) selectively blocks farnesylated, but not geranylgeranylated or myristylated, Ras signal transduction and transformation. Thus, B581 specifically antago-

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; FPTase, Ras protein:farnesyltransferase.

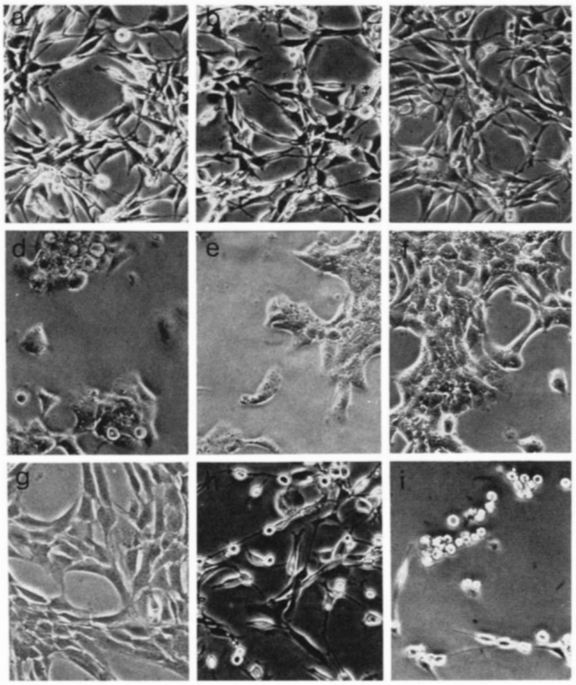

FIG. 1. **B581 induces alterations in the transformed morphology of cells expressing all three lipid-modified forms of oncogenic Ras.** Cultures of cells transformed by the different lipid-modified Ras(61L) proteins were incubated in growth medium alone (*panels a–c*) or growth medium supplemented with 250 μM B581 (*panels d–f*) or 50 μM compactin (*panels h* and *i*). Morphologic alteration was observed within 48 h. *Panels a, d,* and *h*, Ras-F; *panels b* and *e*, Ras-GG; *panels c, f,* and *i*, Myr-Ras; *panel g*, untransformed NIH 3T3 cells.

nizes the growth of Ras-transformed cells via blocking oncogenic Ras activation of critical downstream events.

EXPERIMENTAL PROCEDURES

Ras Constructs and Ras-transformed Cell Lines—The pZIP-*ras*-F, pZIP-*ras*-GG, and pZIP-Myr-*ras* retrovirus expression vector constructs have been described previously (8, 10). pZIP-*ras*-F encodes an oncogenic form of human H-Ras that contains the activating Gln-61 to Leu mutation and is modified by FPTase addition of a 15-carbon farnesyl moiety. pZIP-*ras*-GG encodes a C*AAX* mutant of Ras(61L) (*X* is Leu). This mutant protein is recognized by geranylgeranyl protein transferase I and is modified by addition of a geranylgeranyl moiety (8, 16). pZIP-Myr-*ras* encodes a chimeric protein that contains an NH_2-terminal myristylation signal sequence attached to the nonfarnesylated Ras(61L,186S) mutant, in which the Cys residue of the C*AAX* sequence has been mutated to Ser (10). NIH 3T3 cells stably expressing each Ras(61L) protein were established after transfection and selection in G418-containing growth medium (Dulbecco's modified Eagle's medium, 10% calf serum) (8, 10). To compare the effects of inhibiting farnesylation and all isoprenylation, cells were grown in the absence or presence of either 250 μM B581, an inhibitor of FPTase (prepared in dimethyl sulfoxide) (14), or 50 μM compactin, an inhibitor of the biosynthesis of mevalonate (an obligate intermediate in the synthesis of both farnesyl and geranylgeranyl isoprenoids) (6, 11).

Soft Agar Assay—Colony formation in soft agar was performed essentially as described previously (17). Briefly, single cell suspensions of G418-selected NIH 3T3 cells expressing the indicated Ras(61L) protein were plated in agar at a density of 500 cells/well in 12-well plates in growth medium containing 0.3% agar. Parallel plates contained growth medium supplemented with 100 μM B581. Two weeks after plating, dishes were stained with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue (2 mg/ml) for visualization and quantitation of colonies.

MAPK Assay—Activation of the MAPK pathway in NIH 3T3 cells expressing the different Ras(61L) proteins was determined essentially as described previously (18). Briefly, cells were grown in the absence or presence of B581 (250 μM). After 48 h, total cell extracts were prepared in Laemmli sample buffer and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blot analysis was done using 1B3B9, an anti-$p42^{MAPK}$ rabbit antiserum (gift of M. Weber), to detect the phosphorylated, active and nonphosphorylated, inactive forms of $p42^{MAPK}$/ERK2.

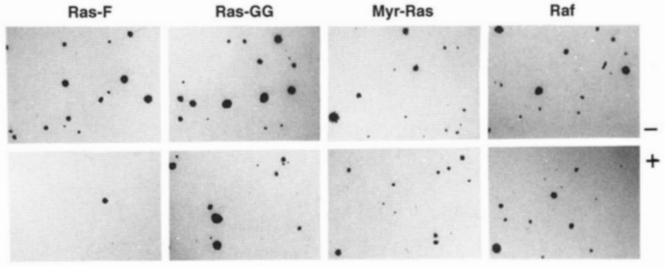

FIG. 2. **B581 preferentially blocks the growth of farnesylated Ras(61L)-transformed cells.** Cells transformed by the indicated oncogene protein were suspended in soft agar in growth medium alone (*top*) or supplemented with 100 μM B581 (*bottom*), as described under "Experimental Procedures." The degree of inhibition was as follows: Ras-F, 89%; Ras-GG, 54%; Myr-Ras, 50%; and Raf, 52%. Colonies were stained and photographed after 14 days.

Activation of Jun and Elk-1 Transcriptional Activity—Transient transfection assays were used to determine the activation of Jun and Elk-1 transcriptional activity as described previously (18). The Gal4-cJun (provided by M. Karin) and Gal4-ElkC (provided by R. Treisman) chimeric expression vectors encode the Gal4 DNA-binding domain linked to the transcriptional activation domains of c-Jun and Elk-1, respectively (19). The 5X Gal-Luc reporter plasmid contains five consensus Gal4 DNA-binding sites upstream of the luciferase gene. Transient transfection assays were done by calcium phosphate coprecipitation using 2.5 μg of reporter plasmid, 250 ng of expression vector, and 250 ng of *ras* or *raf* (p22W-*raf*, encoding an activated form of c-Raf-1) plasmids. Following removal of the transfected DNA, cells were cultured in the absence or presence of 250 μM B581 for 30 h. Luciferase activity was assayed and quantitated as described previously (18).

RESULTS AND DISCUSSION

B581 Preferentially Blocks Growth of Farnesylated Ras-(61L)—We have previously shown that B581, an inhibitor of the Ras FPTase, preferentially blocks farnesylation *versus* geranylgeranylation *in vitro* and *in vivo* (14). To extend these observations to Ras activity in mammalian cells, we first determined the ability of B581 to reverse the transformed properties of Ras-transformed cells.

NIH 3T3 cells expressing the different lipid-modified Ras(61L) proteins were grown in the absence or presence of B581. Like authentic farnesylated Ras-F, both Ras-GG and Myr-Ras also cause morphologic transformation (Fig. 1, *panels a–c*). Surprisingly, we observed that B581 treatment caused a similar alteration in the morphology of all three transformed cell populations (Fig. 1, *panels d–f*). Although the cells exhibited a less refractile and more adherent appearance, this cellular morphology was quite distinct from the morphology of untransformed NIH 3T3 cells (Fig. 1, *panel g*). Instead, the cells showed a relatively flattened and rather cuboidal morphology and appeared as tight clusters of cells with less distinct cell-cell junctions. This B581-induced morphologic change was distinct from the extremely rounded and less adherent phenotype seen with compactin-treated cells (Fig. 1, *panels h* and *i*). Compactin-induced growth inhibition and morphological changes have previously been shown not to be a consequence of blocking Ras function (20). Because both Ras-GG- and Myr-Ras-transformed cells also showed the same alteration in response to B581 treatment as did Ras-F-transformed cells, it suggested that B581 caused a nonspecific effect that was independent of Ras function. We therefore evaluated the consequences of B581 on the growth properties of these cells.

As shown in Fig. 2, B581 preferentially blocked the ability of NIH 3T3 cells expressing Ras-F *versus* cells expressing Ras-GG or Myr-Ras to proliferate in soft agar. As expected, B581 also had limited effect on the soft agar colony formation of cells

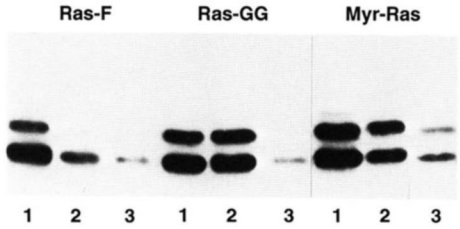

FIG. 3. **MAPK activation is selectively blocked by B581 only in cells transformed by farnesylated Ras-F.** Cells transformed by the indicated Ras(61L) protein were incubated in growth medium supplemented with the following: *lanes 1*, dimethyl sulfoxide (1%) vehicle only; *lanes 2*, 250 μM B581; *lanes 3*, 50 μM compactin. Immunoblotting of $p42^{MAPK}$ shows the inactive, unphosphorylated (*bottom bands*) and active, phosphorylated forms (*upper bands*). The reduced levels of $p42^{MAPK}$ in the *lanes* with compactin-treated cells was a consequence of the Ras-independent growth inhibition due to compactin.

expressing constitutively activated human Raf, which lies downstream of Ras and can cause transformation in a Ras-independent fashion. Therefore, the activity of the FPTase inhibitor B581 in preventing anchorage-independent cellular proliferation of Ras-transformed cells can be ascribed to its ability to specifically interfere with farnesylation of oncogenic Ras.

B581 Specifically Blocks Farnesylated Ras(61L) Activation of MAPKs—Since MAPKs are constitutively activated in Ras-transformed NIH 3T3 cells (1–3), their activation provides an excellent marker for the activation of the Ras signal transduction pathway. We therefore determined whether $p42^{MAPK}$ was constitutively activated in NIH 3T3 cells transformed by the different lipid-modified forms of Ras(61L), and if so, whether B581 could selectively block its activation in cells transformed by Ras-F.

MAPKs are activated by MEK phosphorylation of tyrosine and threonine residues, and the phosphorylated, activated forms show a slower electrophoretic mobility (3). We observed that NIH 3T3 cells transformed by any one of the three lipids can cause constitutive activation of MAPKs (Fig. 3, *lanes 1*). Thus, the transforming function of these alternatively modified Ras proteins is apparently mediated by the same downstream signaling events as authentic Ras(61L).

Treatment of parallel cultures showed that B581 specifically prevented the ability of Ras-F, but not Ras-GG or Myr-Ras, to activate $p42^{MAPK}$ (Fig. 3, *lanes 2*). In contrast, compactin blocked the activity of Ras modified by either a farnesyl or a geranylgeranyl isoprenoid but had no effect on Ras modified by the fatty acid myristate (Fig. 3, *lanes 3*). These results demonstrate that pharmacological inhibition of Ras farnesylation prevents the ability of Ras to transduce signals to activate downstream cellular targets such as MAPKs.

B581 Selectively Blocks Farnesylated Ras(61L) Stimulation of c-Jun and Elk-1 Transcriptional Activity—We next evaluated two downstream events triggered by oncogenic Ras. Elk-1 transcriptional activity is stimulated by MAPK phosphorylation, while the MAPK-related Jun kinase phosphorylates and stimulates Jun transcriptional activity (18, 19, 21). Since both MAPK and MAPK-related Jun kinase activities are triggered by oncogenic Ras (and Raf), we determined the ability of B581 to block Elk-1 and Jun stimulation by the different Ras mutants. We observed that B581 selectively inhibited Ras-F but not Ras-GG, Myr-Ras, or Raf-induced activation of Jun (Fig. 4*A*) and Elk-1 (Fig. 4*B*). These results indicate that B581 showed selective inhibition of signaling induced only by Ras-F.

An essential requirement for a useful *CAAX* peptidomimetic must be its ability to preferentially antagonize FPTase modification of Ras. Since the majority of prenylated proteins in mammalian cells are modified by geranylgeranyl moieties (6, 11), a lack of specificity of a *CAAX* peptidomimetic may result in significant undesirable consequences. We show here that the B581 *CAAX* peptidomimetic compound selectively antagonizes the ability of farnesylated, but not geranylgeranylated, Ras function and that the inhibitory action of this compound is a consequence of specifically blocking the downstream action of oncogenic Ras.

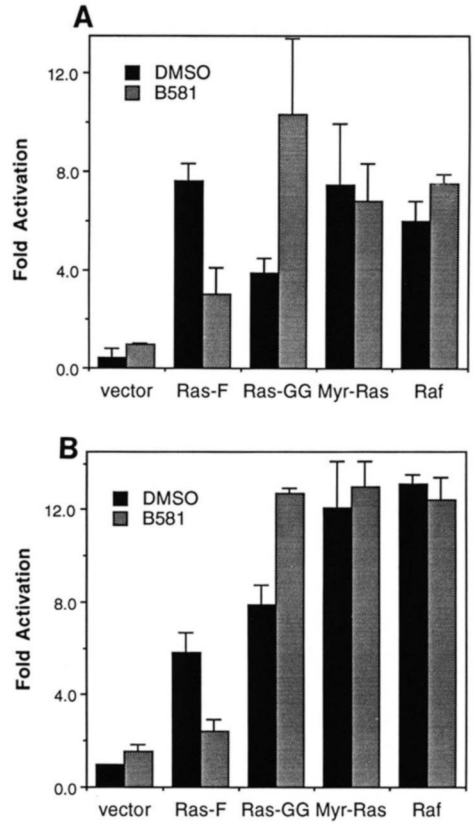

FIG. 4. **B581 selectively blocks Jun and Elk-1 transcriptional activation induced only by oncogenic farnesylated Ras-F.** Transient transfection luciferase assays were done with either Gal4-Jun (*panel A*) or Gal4-ElkC (*panel B*) in the absence or presence of 250 μM B581 as described under "Experimental Procedures." Two experiments were performed in triplicate. Values are normalized to the activity (in relative luciferase units) of vector alone. *DMSO*, dimethyl sulfoxide.

The observation that B581 induced morphological alterations in a farnesylated Ras-independent fashion suggests that the function of other farnesylated proteins may also be perturbed by B581. The farnesylated Ras-related protein, RhoB, which is a regulator of actin cytoskeletal organization (22), is a possible target for B581 action. B581 also blocks the farnesylation of nuclear lamins (14) and may also alter their function (6). However, the continued growth of both Raf- and Myr-Ras-transformed cells in soft agar suggests that neither of these effects is the basis of the ability of B581 to inhibit the growth of the Ras-F-transformed cells. These results suggest that impairment of the function of other farnesylated proteins may not be deleterious to cell viability and provide further evidence that *CAAX*-based FPTase inhibitors may be useful agents for cancer treatment.

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