Peptidomimetic Inhibitors of Ras Farnesylation and Function in Whole Cells*

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The ras protooncogene is involved in regulation of cell growth. Mutations that activate the protein result in uncontrolled cell growth. Ras undergoes a series of post-translational processing events, the first of which, farnesylation, is crucial for the function of the protein. Inhibitors of the farnesyltransferase enzyme are therefore potential candidates for the development of anticancer drugs. Tetrapeptides have been reported to be good inhibitors of this enzyme in vitro. We have synthesized analogs of the tetrapeptide Cys-Val-Phe-Met by replacement of the amino-terminal amide bonds. One inhibitor, B581, is permeable to the cell membrane. In the cell, it inhibits processing of two farnesylated proteins, H-ras and lamin A, but it does not inhibit processing of a geranylgeranylated protein, Rap 1A. Microinjection of B581 into frog oocytes inhibits maturation induced by activated, farnesylated H-ras but not maturation induced by activated, geranylgeranylated H-ras or by progesterone. These results demonstrate that this peptide mimic inhibits farnesylation selectively in the cell. The inhibition of farnesylation results in inhibition of H-ras function.

The ras protooncogene belongs to a group of small GTP-binding proteins that are posttranslationally modified by a prenyl group. Prenylation is followed by proteolytic digestion of the COOH-terminal 3 amino acids and methylation of the now terminal, prenylated cysteine. Two different isoprenyl groups have been found to modify proteins covalently: farnesyl (Ras, lamin B, γ-transducin, and others) or geranylgeranylated (Rap, Rab, Rh0, and other small GTP-binding proteins). Modification by geranylgeranyl (GG)3 is by far the more prevalent (reviewed in Ref. 1).

Several enzymes responsible for prenylation have been isolated and characterized. One of these is FTase, which catalyzes the addition of farnesyl in proteins with the COOH-terminal sequence CAAX (2, 3). In this sequence, A represents any aliphatic amino acid, while X corresponds to Met, Ser, or Ala. Proteins modified with geranylgeranyl fall into three categories based on their COOH-terminal sequence: CAAX (similar to farnesylated proteins), CXC, or CC. Two enzymes responsible for catalyzing the addition of GG to proteins have been isolated and characterized, GGTase I and II. While GGTase I recognizes a CAAX sequence similar to that recognized by FTase, it will only tolerate Leu or Phe at the X position (4). Proteins with the COOH-terminal sequence CXC or CC are prenylated by GGTase II (5).

Oncogenic ras has been linked to 50% of all cases of colon and 90% of cases of pancreas cancer (reviewed in Ref. 6). Single amino acid substitutions at position 12 or 61 are often responsible for Ras activation (i.e. its oncogenicity). By using mutants of the COOH-terminal cysteine (7) or inhibiting prenylation with inhibitors of isoprenoid biosynthesis (lovastatin) (8, 9), it has been shown that the posttranslational modification of Ras by farnesyl is essential for its membrane association and transforming activity. Lack of farnesylation results in a cytoplasmic protein that has lost its growth regulatory function. Thus, inhibition of farnesylation has been postulated to be a good target for the development of anticancer drugs (10). In fact, because of the selectivity of FTase for its own substrate, it seems possible to develop an anticancer drug with high specificity and low toxicity (10).

A number of studies have examined inhibitors of FTase in vitro. These can be divided into two main groups: tetrapeptides with the CAAX motif (11–13) and analogs of FPP (14, 15). A third group of inhibitors with structures not resembling either tetrapeptides or FPP has been described (16–19). None of the latter compounds has activities comparable to that of tetrapeptides or FPP analogs. With tetrapeptides and FPP analogs, potent and highly selective competitive inhibitors of the FTase have been described. However, both have some limitations. For example, peptides are not very membrane-permeable. In the case of FPP analogs, it is not known whether these compounds also affect other steps in cholesterol biosynthesis. FPP is a substrate not only for the farnesyltransferase that prenylates Ras, but also for other enzymes involved in the synthesis of squalene and other prenylation reactions (i.e. synthesis of ubiquinones, heme a) (10). In an effort to avoid non-selective effects related to cholesterol biosynthesis, we have developed inhibitors based on a tetrapeptide structure. This paper describes a tetrapeptide mimic, B581, based on the CVFM structure. This compound inhibits FTase in vitro as well as Ras and lamin A farnesylation in whole cells. Furthermore, the inhibitor is specific for farnesylated versus geranylgeranylated proteins in mammalian cells and frog oocytes.

EXPERIMENTAL PROCEDURES

Proteins—Plasmids containing H-ras or H-ras(61L,CAIL) in the bacterial expression vector pAT were obtained from Dr. C. Der (University of North Carolina). Recombinant protein was prepared from inclusion bodies by standard procedures; inclusion bodies were obtained by centrifugation after breaking bacterial cells by two cycles of freeze-thawing and clarification of the lysate by incubation in 33 mg/ml DNase. The inclusion body pellet was washed twice in 25% sucrose, 1% Triton X-100 and solubilized in 6 mM guanidine hydrochloride, 1 mM EDTA, 50 mM HEPES buffer, pH 7.5, and 1 mM diithiothreitol. The soluble protein was dialyzed overnight against 200 mM NaCl, 5 mM MgCl2, 25 mM HEPES buffer, pH 7.5, 0.1 mM GDP, and 1 mM diithiothreitol. FTase and GGTase I were partially purified from bovine brain as described by Reiss et al. (2).
Inhibitors—CVFM was prepared by standard solution phase peptide synthesis methods using suitably protected, commercially available amino acids. Reduced amide bonds were prepared via reductive amination of N-t-butoxycarbonyl amino aldehydes and the requisite amine HCl salt, using NaCNBH₃ in dimethylformamide/ETOH (21). N-t-Butoxycarbonyl amino aldehydes were prepared by disobutyl aluminum hydride reduction of the corresponding methyl esters. All final compounds were purified by reverse phase high performance liquid chromatography on a C18 column. Stock solutions were prepared in dimethyl sulfoxide.

Prenylation Assay—In vitro prenylation assay was carried out according to Moore et al. (20), with 3 μM recombinant H-ras and 440 nM [3H]geranylgeranyl diphosphate (FTase) or 5 μM recombinant H-ras/61LCAI and 1 μM 1Hgeranylgeranyl dipophosphate (GGTase I). The inhibitors were diluted in assay buffer, and the assay mixture was incubated 15 min at 37°C.

Preparation of Cell Lysate—A cell lysate was prepared by hypotonic shock of B-ras(12V)-transformed NIH3T3 fibroblasts. Cells from a confluent 10-cm plate were harvested by trypsinization and centrifugation. The cell pellet was resuspended in 400 μl of water and incubated on ice for 15 min, followed by homogenization with a Dounce homogenizer. The suspension was centrifuged at 300,000 x g for 20 min, and the supernatant was used as cell lysate.

Protein Processing Assay—H-ras(61L)-transformed NIH3T3 fibroblasts were obtained from Dr. C. Der. After 24 hr of treatment with 50 μM lovastatin (courtesy of Dr. A. Alberts, Merck Research Laboratories) or the indicated concentrations of B581, cells were lysed in 1% Nonidet P-40, 5 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.1 mM N-tosyl-l-phenylalanine chloromethyl ketone, 0.1 mM N-tosyl-l-lysinine chloromethyl ketone, and 1 mM phenylmethylsulfonyl fluoride. The lysate was separated by centrifugation and the supernatant used as cell extract. Lamin A was extracted from nuclear pellets (22) and resuspended with 50 μl of 10% SDS. Total protein was separated by SDS-polyacrylamide gel electrophoresis in 15% (Ras, Rap) or 8% (lamins) acrylamide gels. After transfer to Immobilon P membrane (Millipore), the blots were probed with polyclonal antibody to Rap 1/Krev (Santa Cruz Biotechnology), or IE, LA069 mouse monoclonal antibody to H-ras (Quality Biotech), rabbit monoclonal antibody to lamin A and C (courtesy of Dr. F. McKeon, Harvard University). All Western blots were developed using enhanced chemiluminescence reagents (Amersham Corp.).

Fig Oocytes—Groups of 20 Xenopus laevis oocytes were microinjected with 10 nl solution containing protein and vehicle (0.5% Me₂SO) or protein and inhibitor. The oocytes were incubated in 96-well plates at 27°C, supplemented with 5% horse serum, with or without 6.4 μM lovastatin (courtesy of Dr. A. Alberts, Merck Research Laboratories) or the indicated concentrations of B581, cells were lysed in 1% Nonidet P-40, 5 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.1 mM N-tosyl-L-lysine chloromethyl ketone, 0.1 mM N-tosyl-l-lysinine chloromethyl ketone, and 1 mM phenylmethylsulfonyl fluoride. The lysate was separated by centrifugation and the supernatant used as cell extract. Lamin A was extracted from nuclear pellets (22) and resuspended with 50 μl of 10% SDS. Total protein was separated by SDS-polyacrylamide gel electrophoresis in 15% (Ras, Rap) or 8% (lamins) acrylamide gels. After transfer to Immobilon P membrane (Millipore), the blots were probed with polyclonal antibody to Rap 1/Krev (Santa Cruz Biotechnology), or IE, monoclonal antibody to lamin A and C (courtesy of Dr. F. McKeon, Harvard University). All Western blots were developed using enhanced chemiluminescence reagents (Amersham Corp.).

RESULTS AND DISCUSSION

The most active tetrapeptide inhibitor of farnesytransferase reported to date corresponds to the sequence CVFM, which has an IC₅₀ = 60 nM in vitro (11). CVFM is not modified by the enzyme, apparently due to the presence of both an aromatic residue and a free amino terminus (11). Therefore, CVFM is not a substrate but a true inhibitor. These properties make it a good candidate for in vivo inhibition of farnesylation. However, in general, peptides are neither stable nor permeable enough through cell membranes to be used in vivo. In an effort to increase both stability and permeability, we have modified the CVFM molecule by replacing one or two NH₂-terminal peptide bonds to produce compounds B515 and B581, respectively (Fig. 1). These modifications resulted in a 2-fold increase in activity as inhibitors of farnesyltransferase in comparison to CVFM (Table I). While their selectivity against FTase is not as great as that of CVFM, these inhibitors are still more than 30-fold more active against FTase than GGTase. Since B581 has a slightly better selectivity for FTase than B515, B581 was used in all subsequent studies.

One important characteristic of a cell-based inhibitor is its stability. To test resistance to proteolysis, B581 at 10 μM concentration was incubated in a cell lysate expected to contain many proteolytic enzymes (see "Experimental Procedures"). After incubation, the compound in the cell lysate was diluted and evaluated as inhibitor in a farnesyltransferase assay. The results (Fig. 2) indicate that B581 is not only stable to proteolysis, but its activity is not affected by the presence of cell components. Under the same conditions, CVFM becomes completely...
inactive (Fig. 2). Analysis of prenylated products by TLC indicates that B581 is not modified by either FTase or GGTase (data not shown). Thus, B581 is a true inhibitor of farnesyltransferase in vitro.

Although tetrapeptides are not very permeable through plasma membranes, the structural modifications introduced in B581 might increase its cell permeability with respect to CVFM. To determine permeability and stability, NIH3T3 fibroblasts transformed with c-H-ras(61L) were incubated for 24 h in the presence or absence of 10–500 μM B581. A cell extract was analyzed by Western blotting for posttranslational protein processing. The effect of B581 was compared to the effect of the known prenylation inhibitor lovastatin. B581 inhibits H-ras processing in a dose-dependent manner (Fig. 3A). This is indicated by the appearance of a slower migrating band corresponding to non-prenylated, unprocessed H-ras. The IC50 for inhibition of processing is approximately 50 μM, and the effect is comparable to that induced by treatment with lovastatin. The fact that the concentration necessary to inhibit processing by 50% is more than 1000-fold higher than the concentration needed to inhibit farnesylation in vitro is probably the result of low or limited permeability. However, although the in vitro assay indicated proteolytic stability (Fig. 2), other factors such as binding or compartmentalization might contribute to lower activity in the cell.

In contrast to the processing of Ras, analysis of posttranslational processing of an endogenous geranylgeranylated protein, Rap 1A, showed that processing of this protein was not affected by B581 (Fig. 3B). On the other hand, treatment with lovastatin did inhibit Rap 1A processing, as indicated by the appearance of a more slowly migrating protein. These results demonstrate that the inhibitor B581 selectively inhibits farnesylation and does not affect geranylgeranylation. Since the majority of isoprenylated proteins in the cell are modified by geranylgeranylation, these results support the idea that a specific farnesylation inhibitor like B581 could be developed as a potential drug against Ras-dependent cancer.

Studies on another farnesylated protein, nuclear lamin A (23), also indicated that posttranslational processing was inhibited by B581 (Fig. 3C). The inhibition was concentration-dependent, with an IC50 higher than 100 μM. These results indicate that B581 is capable of blocking farnesylation of other non-Ras substrates by FTase. It has been suggested that lamin A is prenylated in the nucleus (24), which indicates that B581 is capable of permeating not only the cell membrane, but also the nuclear membrane. It is possible that the higher concentration of inhibitor needed to inhibit lamin A farnesylation (as opposed to inhibition of H-ras farnesylation) is due to the need to permeate the nuclear membrane.

Frog oocytes were used to determine whether inhibition of Ras farnesylation affects Ras function. Microinjection of activated, recombinant H-ras into X. laevis oocytes induces meiotic maturation (25). This process is characterized by the appearance of a white spot in the animal pole, corresponding to the breakdown of the germinal vesicle. As in mammalian cells, farnesylation is necessary for Ras function in oocytes: microinjection of oocytes with lovastatin or CAAX tetrapeptides inhibits GVBD (8, 26). Thus, the oocytes possess the adequate posttranslational processing machinery. On the other hand, oocytes undergo maturation through at least two different pathways: Ras-dependent and Ras-independent, progesterone-mediated (27). The Ras-dependent pathway has been compared to mammalian cell transformation (28). This provides us with a system in which we can show selectivity as well as possible side effects of the inhibitor.

Oocytes were microinjected with 74 ng of H-ras(61L). The appearance of GVBD was observed 3–4 h after microinjection, with 50% of the oocytes showing GVBD in 4.5 h (Fig. 4A, closed circles). When H-ras(61L) and B581 were coinjected, the rate of appearance of GVBD was decreased in a dose-dependent manner: 2.5 or 5 pmol of B581 delayed the appearance of GVBD by about 3 h (Fig. 4A, crosses and triangles). In the presence of 25 pmol of B581, maturation was not apparent until 7 h after microinjection, i.e., 4 h later than in the absence of inhibitor (Fig. 4A, open circles). Control, mock-injected oocytes did not undergo maturation, and oocytes injected with B581 alone had the same appearance as mock-injected oocytes. To demonstrate that no other functions are affected, oocytes injected with B581 or vehicle were incubated in 2 μg/ml progesterone. The result shown in Fig. 4B indicates that B581 had no effect on progesterone-induced oocyte maturation.

To test the selectivity of the inhibitor, activated H-ras with the 3 COOH-terminal amino acids mutated to Ala-Ile-Leu was injected into oocytes. In this case, the protein is expected to be modified by GG instead of farnesyl. Oocytes injected with H-ras(61L,CAIL) undergo maturation in 4 h (Fig. 4C), similar to oocytes injected with H-ras(61L). However, in this case coinjection of Ras with B581 did not result in inhibition of the oocyte maturation process. Inhibition of geranylgeranylation

![Fig. 3. Effect of B581 on posttranslational processing of prenylated proteins.](image-url)
processes associated with oocyte maturation are affected.

In summary, the data presented in this paper demonstrate that peptidomimetic inhibitors of farnesylation, such as B581, inhibit farnesylation of proteins in whole cells. Such inhibitors should provide tools to answer questions regarding the role of prenylated proteins in cell function. Important characteristics of B581 are its stability, membrane permeability, and selective inhibition of farnesylation over other prenylation reactions. Furthermore, inhibition of Ras farnesylation by B581 results in inhibition of Ras function in whole cells. It is likely that structural modifications to improve the membrane permeability of B581 will result in a more potent inhibitor.

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Fig. 4. Effect of B581 on the time course of Ras-induced X. lutea oocyte maturation. At the indicated times after microinjection, oocytes were scored for the appearance of GVBD. Data is expressed as percent of total number of oocytes injected. A, oocytes microinjected with 74 ng of H-ras(61L) and vehicle (○) or 2.5 ng (●), or 25 (▲) pmol of B581. B, oocytes microinjected with vehicle (●) or 2.5 ng (●), or 25 (▲) pmol of B581. After microinjection, the oocytes were transferred to ND-96 with 5% horse serum and 6.4 μM progesterone. C, oocytes microinjected with 36 ng of H-ras(61L,CAIL) and vehicle (●) or 2.5 ng (●), or 25 (▲) pmol of B581.

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