Evidence for a High Affinity, Saturable, Prenylation-dependent p21\textsuperscript{Ha-ras} Binding Site in Plasma Membranes*

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Oncogenic p21\textsuperscript{ras} proteins can only exert their stimulation of cellular proliferation when plasma membrane-associated. This membrane association has an absolute requirement for post-translational modification with isoprenoids. The mechanism by which isoprenoids participate in the specific association of p21\textsuperscript{ras} with plasma membranes is the subject of this report. We present in \textit{vitro} evidence for a plasma membrane binding protein for p21\textsuperscript{ras} that can recognize the isoprenoid substituent and, therefore, may facilitate the localization of p21\textsuperscript{ras}.

Post-translational modification with isoprenoids results in considerable structural diversity at the carboxyl terminus of many proteins. Four such carboxyl-terminal structural motifs have been identified (for review see Ref. 1). These are farnesylated-methylated cysteine, geranylandylated-methylated cysteine, digeranylgeranylated vicinal cysteines of the -CC rab proteins, and digeranylgeranylated residue-interrupted cysteines of the -CXC rab proteins. The carboxyl-terminal cysteine of the -CXC rabs is methylated, whereas the carboxyl-terminal cysteine of the -CC rabs is not. Since these modifications always increase the hydrophobic character of the substituted proteins, it is generally assumed that they are involved in the association of proteins so modified with lipid bilayer membranes. This assumption is supported by the observation that most prenylated proteins are bound to cellular membranes, at least under some physiological conditions.

A more recent hypothesis for the function of protein prenylation that is more in keeping with the observed structural diversity and membrane specificity is that these lipid modifications also serve to mediate protein-protein interactions (2). A well studied example of this function for protein prenylation is the heterodimeric association of rab proteins with GDP dissociation inhibitor molecules to form soluble complexes that appear to be dependent on the digeranylgeranylation of the rab proteins (3). Another recent example from our laboratory is the mechanism of endoproteolytic cleavage of the farnesylated prelamin A molecule, which is mediated by an enzyme that possesses a specific farnesyl binding site (4).

It has been noted that it is possible that even membrane-associated prenylated proteins may utilize a polyisoprenoid-dependent protein-protein interaction for membrane binding (2). Recognition of the polyisoprenoid and other structural elements of the protein by membrane receptors could confer the necessary localization of particular prenylated proteins to particular subcellular compartments. Sequestration of the polyisoprenoid would be important in preventing nonspecific association of the protein with inappropriate membranes.

Farnesylated proteins are found in a number of cellular compartments (1 including plasma membrane (p21\textsuperscript{ras}, γ-tubulin, peroxisomes (PXF) and nuclear lamin A proteins). For the naturally occurring forms of N, H, and K p21\textsuperscript{ras}, it is clear that plasma membrane localization shows an absolute requirement for farnesylation (5), yet the occurrence of farnesylated proteins in other compartments suggests the existence of some high affinity plasma membrane receptor that can recognize not only the isoprenoid but other structural features of the protein as well. Studies on site-directed mutagenetic alterations of p21\textsuperscript{ras} proteins are consistent with this concept and have clearly identified the hypervariable region of p21\textsuperscript{ras} as a second domain that specifies its plasma membrane localization (6). In this report we present quantitative in \textit{vitro} binding studies that confirm the evidence of a high affinity plasma membrane receptor for p21\textsuperscript{ras}.

MATERIALS AND METHODS

NIH3T3 Mouse Fibroblast Culture and Plasma Membrane Isolation—NIH3T3 mouse fibroblast cultures were grown in Dulbecco’s modified Eagle’s medium (high glucose) (Novo-Tech, Inc.) supplemented with 10% fetal bovine serum (v/v) (Life Technologies, Inc.), 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml amphotericin B. Twenty-four h before harvesting the cells for plasma membrane isolation, culture media was supplemented with 8 μg/ml lovastatin to deplete the association of endogenous p21\textsuperscript{ras} with the plasma membrane.

A highly enriched plasma membrane fraction from NIH3T3 cells was isolated as described (7). A protease inhibitor mix (1 mM each of antipain, leupeptin, aprotinin, chymostatin, pepstatin A, bestatin, and K p21 ras, CVLL-p21Ha-ras and Lamin B; Purification of Recombinant Proteins and Isolation of p21\textsuperscript{Ha-ras}.—Recombinant baculoviruss-expressing p21\textsuperscript{Ha-ras} with a histidine tag affixed to its N terminus was a gift from Dr. Sandra L. Hoffmann (University of Texas Southwestern Medical Center, Dallas, TX). A BamHI/Sall fragment containing full-length coding sequence of p21\textsuperscript{Ha-ras} and a BamHI/HindIII fragment containing full-length sequence of p21\textsuperscript{Ha-ras} were cloned into compatible cloning sites of pBlueBacHis2 vector (Invitrogen, San Diego, CA). The constructs were cotransfected with Bac-N-Blue baculovirus DNA into sf-9 cells (Invitrogen) with the aid of a cationic liposome-mediated transfection kit (Invitrogen) employed according to the manufacturer’s instructions. The recombinant baculoviruses were screened for and isolated in sf-9 cells as described (10). A single virus clone for each of the proteins was chosen for all further experimentation. Approximately, 5 × 10⁸ sf-9 cells (Invitrogen)75-cm² flask were infected (~1 plaque-forming unit/cell) with recombinant baculoviruses derived from p21\textsuperscript{Ha-ras}, p21\textsuperscript{Ki-ras}, and p21\textsuperscript{N-ras} constructs, respectively. The infected cells were grown in complete TMN-FH media (Invitrogen) supplemented with 10 μg/ml gentamicin at 27 °C for 48 h. The infected sf-9 cells were collected by centrifugation and after several washings with...
PBS, were processed for membrane and cytoplasmic fractions isolation (11, 12). Both cytoplasmic- and membrane-associated forms of recombinant p21_Ha-ras and membrane-associated forms of p21_Ki-ras and p21_N-ras were purified via metal affinity chromatography Xpress System (Invitrogen) and imidazole elution (50–75 mM). Recombinant baculovirus expressing p21_Ha-ras was a gift from Dr. Paul Kirschmeier, Schering-Plough Research Institute, Kenilworth, N.J. Sf-9 cells were infected with recombinant baculovirus-expressing CVL-p21_Ha-ras as above, and the resultant protein was purified as described (10). A highly purified preparation of lamin B was obtained from rat liver using published procedures (13).

Binding of 125I-labeled p21_Ha-ras to NIH3T3 Plasma Membranes and Competition Assays— Aliquots of plasma membranes (100 µg of protein) were incubated with varying concentrations of 125I-labeled p21_Ha-ras (5–150 nM) with or without a 100-fold excess of unlabeled p21_Ha-ras in a total volume of 100 µl. The incubation medium consisted of phosphate-buffered saline containing 5 mM MgCl₂, 30 µM guanosine 5'-diphosphate, 0.1% bovine serum albumin, 0.5% NaCl, and 0.8% Triton X-100. The binding of the radioactive ligand was allowed to proceed for 1 h at 25 °C. Incubations were terminated by the addition of ice-cold PBS containing 0.8% Triton X-100 and 0.1% bovine serum albumin. The unbound radioactivity was removed via filtration of the reaction mixture through Whatman glass microfiber filters. The filters were washed ≥6 times with PBS containing 0.8% Triton X-100 and 0.1% bovine serum albumin and air-dried, and radioactivity was counted in a gamma counter.

A time course experiment for binding was performed for 15, 30, 45, 60, and 75 min, utilizing the optimal conditions outlined above. Saturation of the binding was observed after 45 min, and therefore all experiments were conducted for 1 h. To determine reversibility of binding, matched samples of plasma membranes (100 µg of protein) were incubated with 125I-labeled p21_Ha-ras (125 nM), and the binding of the radioactive ligand was allowed to proceed at 25 °C. After a 1-h incubation, a 100-fold excess of unlabeled p21_Ha-ras was added to one set of samples, and incubation was continued for an additional 1 h. Incubations were then terminated by the addition of ice-cold PBS containing 0.8% Triton X-100 and 0.1% bovine serum albumin. The reaction mixture was then centrifuged at 13,000 × g for 15 min, and the difference between the membrane-bound counts in the presence and absence of excess unlabeled p21_Ha-ras was determined.

To study the effect of different competitors (non-farnesylated p21_Ha-ras, bovine serum albumin, p21_N-ras, p21 Ki-ras, CVL-p21_Ha-ras, rat liver lamin B, LLGNSSPRTQSPQSCNfarnesyl, and N-acetyl farnesyl methyl cysteine) on binding of iodinated p21_Ha-ras to plasma membranes, varying concentrations (5 nM to 5 µM) of competitors were added with 125I-labeled p21_Ha-ras (50 nM) under binding conditions outlined above.

Nonlinear regression analysis of saturation binding and competitive inhibition data were performed with SigmaPlot (Jandel Scientific, San Rafael, CA). Scatchard plot analysis and calculations of IC₅₀ values were performed as described (14) with the linear regression performed with Sigma Plot.

To evaluate whether the binding of p21_Ha-ras to plasma membrane is dependent on p21_Ha-ras being bound to GTP or GDP, the binding studies were carried out in the presence of 30 µM GDP or 25 µM GTP·S (BioL) under the conditions outlined for binding of 125I-labeled p21_Ha-ras to NIH3T3 plasma membranes above. These concentrations of unbound ligand are approximately 1,000-fold excess to the concentration of GDP-loaded p21_Ha-ras, which gives half-maximal binding to plasma membranes. Binding reaction mixtures were incubated at 37 °C for 100 min in the absence of membranes to allow nucleotide exchange to occur (15) and transferred to a 25 °C water bath, and the binding reaction was initiated by the addition of membranes.

Ligand Blotting Assays—Ligand blots were performed by a previously described method (16) with some modifications. Approximately 25 µg of NIH3T3 plasma membrane proteins were separated via SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The blots were incubated in PBS containing 0.3% Tween, 0.5% Triton X-100, 5 mM MgCl₂, 30 µM guanosine 5'-diphosphate, 0.1% bovine serum albumin, and 125I-labeled p21_Ha-ras (50 nM) with or without a 100-fold excess of unlabeled p21_Ha-ras with constant shaking at 25 °C. After an 18-h incubation, blots were washed (>5 times) with PBS containing 0.3% Tween and 0.5% Triton X-100, air-dried, and exposed to Kodak X-Omat AR film at −70 °C.

Binding of Iodinated p21_Ha-ras with Trypsin-treated Plasma Membranes—To determine if the binding of iodinated p21_Ha-ras is to the protein component(s) of the plasma membrane, NIH3T3 plasma membranes were pretreated with trypsin (10 µg/ml) for 30 min at 25 °C followed by the addition of protease inhibitor mix. For control experiments, trypsin was inactivated with protease inhibitor mix and then added to the binding reaction mixture. For other series of experiments, plasma membrane proteins were also inactivated by incubation at 100 °C for 5 min. The binding studies were carried out exactly as above.

Other Assays—Protein concentrations were determined by BCA method (Pierce). N-Acetyl farnesyl methyl cysteine was prepared by acid-catalyzed methylation of commercial N-acetyl farnesylcysteine (Calbiochem) as described previously (17). Gas-liquid chromatographic analysis of the radiolabeled isoprenoid group of baculovirus-generated p21_Ha-ras was done after Raney nickel cleavage of immunoprecipitated [3H]mevalonate-labeled protein, as described previously (18).

RESULTS

Saturation Binding of Farnesylated p21_Ha-ras to 3T3 Cell Plasma Membranes—Farnesylated, geranylgeranylated, and nonprenylated p21_Ha-ras proteins were synthesized in the baculovirus insect cell expression system, and the proteins were purified to homogeneity as described under "Materials and Methods" (Fig. 1). All of the baculovirus-generated p21_Ha-ras proteins showed immunoreactivity with Y13–259 antibody in Western blots (data not shown). Synthesis of protein in the presence of [3H]mevalonate followed by immunoprecipitation gave rise to labeled material from membranes. This material was analyzed for farnesylation by Raney nickel cleavage followed by radiolabeled gas-liquid chromatography and radioimmunoassay (Fig. 2) as described previously (18). These results further demonstrate that we have prepared bona-fide p21_Ha-ras in sf-9 insect cells, as has also been reported elsewhere (11, 19–21).

NIH3T3 cells were pretreated with lovastatin for 24 h before harvest for membrane preparation to decrease any endogenous p21_Ha-ras occupancy of potential farnesylase-dependent binding sites. An enriched plasma membrane fraction from lovastatin-treated NIH3T3 cells was prepared by means of discontinuous Ficoll and sucrose density gradients. Marker enzyme analysis of the plasma membrane fraction showed that the specific activity of 5'-nucleotidase was 12.5-fold enriched in this fraction compared with the initial homogenate. Based on glucose-6-phosphatase activity, the cross-contamination of the plasma membrane fraction with microsomes was approximately 11%.

We examined the kinetics of binding of the purified, 125I-labeled p21_Ha-ras to the NIH3T3 plasma membranes, subtracting out nonspecific binding estimated from the binding in the presence of excess unlabeled ligand. The results (Fig. 3) are consistent with a saturable binding site for p21_Ha-ras, presumably a re-
obtain any inhibition of farnesylated p21\(^{\text{Ha-ras}}\) by the nonfarnesylated protein at concentrations as much as 1,000-fold higher than the farnesylated ligand (Fig. 6). On the other hand, unlabeled, fully processed N, H, or K p21\(^{\text{ras}}\) prepared from sf-9 cells were all efficient competitive inhibitors.

The farnesylation requirement for receptor binding is indicative of recognition of the polyisoprenoid moiety. To examine this point further, we synthesized N-acetyl-S-farnesyl methyl cysteine, an analogue of the carboxyl-terminal amino acid residue of the p21\(^{\text{ras}}\) proteins. This compound is a weak competitive inhibitor of p21\(^{\text{Ha-ras}}\) binding to the receptor (Table I). Competitive inhibition was also observed with another farnesylated protein, lamin B, and with an S-farnesyl cysteine methyl ester peptide corresponding to the carboxyl-terminal 15 amino acid residues of prelamin A (Table I). These results indicate that although the receptor recognizes the polyisoprenoid moiety, binding is also mediated by another domain on the p21\(^{\text{ras}}\) protein.

It has been reported that transfection of 3T3 cells with geranylgeranylated p21\(^{\text{Ha-ras}}\) (CAAX = CVLL) results in a membrane-associated protein that is transforming when oncogenic but inhibits cellular growth p21\(^{\text{Ha-ras}}\) when wild type (22). We considered the possibility that this observation might reflect some difference in the interaction of this reported (22) geranylgeranylated protein and the wild-type farnesylated protein with the receptor. Competitive inhibition studies with this CVLL mutant of p21\(^{\text{Ha-ras}}\), previously shown to be geranylgeranylated and to produce growth inhibition, were performed. The results (Fig. 6) indicate that the, presumably, geranylgeranylated p21\(^{\text{Ha-ras}}\) has an affinity for the receptor that is comparable to that of the wild-type p21\(^{\text{ras}}\) proteins. These results suggest that the p21\(^{\text{ras}}\) receptor does not exhibit a strong discrimination between farnesyl and geranylgeranyl substituents.

**FIG. 2.** Farnesylation of membrane-associated p21\(^{\text{Ha-ras}}\) expressed in sf-9 cells. Infected cells were incubated at room temperature overnight with [\(^{3}H\)]mevalonolactone (200 \(\mu\)Ci/ml) in the presence of 25 \(\mu\)M lovastatin. Labeled p21\(^{\text{Ha-ras}}\) derived from the membrane fraction of infected sf-9 cells was treated with Raney nickel to release the isoprenoid moiety, which was then analyzed by gas-liquid chromatography and radiodetection (B). The peaks for p21\(^{\text{Ha-ras}}\) were identified by comparison of retention time values with the ones generated from Raney nickel-treated synthetic farnesyl (peak 1) and geranylgeranyl cysteine methyl esters (peak 2) (A). Recombinant p21\(^{\text{Ha-ras}}\) released only the farnesyl group (A, peak 1). FID, flame ionization detector; cps, counts/s; stds, standards.

**DISCUSSION**

Plasma membrane localization of p21\(^{\text{ras}}\) has been shown to be critical for both normal and oncogenic signaling activities of these proteins (23). Elegant mutant expression studies on recombinant p21\(^{\text{ras}}\) have demonstrated the structural requirements for N, H, and K p21\(^{\text{ras}}\) protein localization to cell plasma membranes (5, 24). Plasma membrane association of p21\(^{\text{Ha-ras}}\) or p21\(^{\text{N-ras}}\) requires post-translational modification of the protein by two alkyl groups (24). The expression studies indicate
that post-translational modification by acylation (palmitoylation at Cys-181 or Cys-185 or N-terminal myristoylation) and/or prenylation (farnesylation or geranylgeranylation) produces at least some association of the dialkylated protein with plasma membranes. Mutants of p21\textsuperscript{Ha-ras} that only undergo farnesylation do not exhibit plasma membrane association of p21\textsubscript{ras} as detected by immunofluorescence. The carboxyl terminus of p21\textsuperscript{Ha-ras} thus possesses a dialkyl structure that appears to be required for its plasma membrane binding. It has been noted (24) that the close proximity of the N terminus and C terminus of p21\textsuperscript{ras} would cause myristoylated and carboxyl-terminal-monolipidated p21\textsuperscript{ras} to present a structure comparable to that of the wild-type protein. The recognition site for this structure in the plasma membrane has been hypothesized to be a receptor or docking protein that mediates assembly of p21\textsuperscript{ras} into the membrane where it is stabilized by nonspecific lipid-lipid interactions.

The studies reported here, confirm and extend these ideas. Based on the amount of protein per cell (~1 mg/10^7 cells) and the fraction of total cellular protein found in the plasma membrane-enriched fraction (~5%) used in our binding studies, we estimate that there are approximately 5 × 10^5 p21\textsuperscript{ras} receptors/cell. This can be compared with the ~5 × 10^5 sites/cell reported for transmembrane-bridging mitogenic peptide receptors such as those for platelet-derived growth factor and epidermal growth factor (25), which are believed to transduce growth signals through p21\textsuperscript{ras}. If nothing else, this number suggests that the total number of such receptors is relatively small and, therefore, inconsistent with being a lipid or a ubiquitous membrane protein. The \( K_d \) (25 nM) we observe for p21\textsuperscript{Ha-ras} binding can be compared with that seen for another farnesylation-dependent protein-protein interaction of p21\textsuperscript{ras}. In vitro binding of p21\textsuperscript{Ha-ras} to human SOS1 has been shown to be absolutely dependent on farnesylation and exhibits 50% saturation at 200 nM (26). In this comparison, the farnesylation-dependent binding we have observed appears to be at relatively high affinity.

The ligand blot data we report are also consistent with a farnesyl group-dependent binding site for p21\textsuperscript{ras} in plasma membranes. Other p21\textsuperscript{ras} binding activities, with the exception of the cytosolic hSOS1 protein noted above, do not exhibit such a requirement for farnesylation. For example, it has been reported (27) that the 21–24-kDa protein caveolin has p21\textsuperscript{Ha-ras} binding activity. Caveolin was shown not to have an absolute
Ha-ras capable of binding p21 were separated by SDS-polyacrylamide gel electrophoresis and trans-
NIH3T3 plasma membrane proteins.

Nonfarnesylated p21 were visualized by autoradiography. The binding activity we have identified is certainly consistent with this hypothesis. A second possibility is that the p21 receptor represents a terminal destination for p21. It should be noted in this regard that the Kᵢ for the p21 receptor is much lower than that reported (28) for binding of the alkylation carboxymethylated model p21(Kᵢ = 480 nM) to lipid vesicles, and therefore p21 receptors could be occupied even though surrounded by lipid bilayer. It is also possible that both the p21 receptor and the lipid bilayer are terminal destinations for p21 but mediate different functions.

The reversibility of binding of the farnesylated p21 as well as the negatively cooperative binding kinetics observed with GDP and GTP-S-loaded p21 has been consistent with the possibility that the association of p21 with the plasma membrane may be regulated. The sigmoidal kinetics observed when the p21 is GTP-loaded suggests that the receptor may exist as a homodimer in the membrane. In this regard, it is of interest to note that a pool of farnesylated cytosolic p21 has been reported to exist in 3T3-L1 cells (29).

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<table>
<thead>
<tr>
<th>Competitor</th>
<th>IC₅₀ (nM)</th>
<th>Kᵢ or Kᵢ (mean ± S.E.)</th>
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<tr>
<td>Unlabeled farnesylated p21 ras</td>
<td>61.6</td>
<td>27.7 ± 3.4</td>
</tr>
<tr>
<td>p21 ras</td>
<td>106.4</td>
<td>47.9 ± 7.3</td>
</tr>
<tr>
<td>CVLL-p21 ras</td>
<td>135.5</td>
<td>61.1 ± 8.2</td>
</tr>
<tr>
<td>N-acetyl farnesyl methyl cysteine</td>
<td>183.6</td>
<td>52.8 ± 13.3</td>
</tr>
<tr>
<td>LLGNSSPRTSPQN(acetylated)</td>
<td>379.4</td>
<td>171.0 ± 23.2</td>
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<tr>
<td>Bovine serum albumin</td>
<td>No competition</td>
<td></td>
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requirement for farnesylation of p21 ras to bind it, whereas our binding activity does. We also tested our ligand blot with parallel immunoblots with anti-caveolin and did not find co- 
migration of caveolin with p21 ras binding activity (data not shown).

We have recently reported a kinetic analysis (4) of the prelamin A endoprotease that demonstrates that this enzyme functions through specific binding of the farnesyl group of prelamin A as well as another domain on that protein. The studies reported here are consistent with an analogous p21 ras receptor that recognizes the lipid moiety but, based on the weak competition for binding of N-acetyl farnesyl cysteine methyl ester (which is also detectable in the ligand blots) and the prelamin A peptide, recognizes other structural features of p21 ras as well.

A plasma membrane receptor for p21 ras that recognizes the protein at least in part through its isoprenoid substituent is, as noted above, consistent with a large body of literature regarding the effect of site-directed mutations on p21 ras localization to the plasma membrane. There are, however, at least two functional interpretations of such a binding protein. Hancock and co-workers (24), have suggested that H and N p21 ras assemble into plasma membranes through initial recognition of two alkyl groups by a docking protein. The bound protein is then unloaded into the membrane bilayer where it is stabilized by hydrophobic interaction of the alkyl groups with the lipid bilayer. The binding activity we have identified is certainly consistent with this hypothesis. A second possibility is that the p21 ras receptor represents a terminal destination for p21 ras. It should be noted in this regard that the Kᵢ for the p21 ras receptor is much lower than that reported (28) for binding of the alkylated carboxymethylated model p21(Kᵢ = 480 nM) to lipid vesicles, and therefore p21 ras receptors could be occupied even though surrounded by lipid bilayer. It is also possible that both the p21 ras receptor and the lipid bilayer are terminal destinations for p21 ras but mediate different functions.

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