Introduction

p21\textsuperscript{ras} proteins are members of an expanding superfamily of small guanine nucleotide-binding proteins (G proteins) which are involved in the regulation of many different aspects of cell function [1]. Mutations of ras genes which lead to the expression of oncogenically activated ras proteins, are found relatively frequently in human cancers. The incidences being particularly high (50–90%) in colonic and pancreatic carcinomas [2]. Although the exact role played by oncogenic ras proteins remains unclear, in colonic cancer ras oncogenes are considered to co-operate with other genetic changes to promote development of the tumour [3]. Inhibitor that could interfere with oncogenic p21\textsuperscript{ras} function might therefore provide a chemotherapeutic approach for treatment of some cancers.

To express their biological activity, ras proteins have to become associated with the cell plasma membrane. Mutations which block this process have been shown to inhibit the ability of oncogenic ras to cause cellular transformation [4, 5]. Membrane association of Ras occurs following a complex sequence of post-translational events including isoprenylation, C-terminal proteolysis and carboxymethylation. These modifications of the Ras precursor are targeted on the C-terminal-CAAX motif and it has been demonstrated that farnesylation of the cysteine residue (Cys-186) is essential for the biological activation of all three ras proteins [6–8].

On the basis of these observations it became evident that inhibitors either of isoprenoid biosynthesis or alternatively of enzymes involved in the farnesylation of the ras proteins, might represent anti-ras drugs. Inhibitors of isoprenoid biosynthesis such as mevinolin, which acts at the level of hydroxymethylglutaryl-CoA (HMG-CoA) reductase, have been shown to inhibit Ras processing and membrane association [6]. However, since the high concentration of these compounds required to inhibit farnesylation also inhibit several important metabolic pathways and generally suppress protein isoprenylation, they lack sufficient selectivity. Consequently, development of inhibitors of p21\textsuperscript{ras} farnesyltransferase may represent a more satisfactory approach to find anti-ras drugs.

Recently, enzymes with farnesyltransferase activity have been identified in several mammalian tissues [9–11], and a major form present in rat brain has been purified to homogeneity [9]. A structure for the rat brain enzyme involving α and β subunits has been proposed [12], and a cDNA encoding the peptide-binding β-subunit has been cloned [13]. The α-subunit of farnesyltransferase appears to be shared by another prenyltransferase, which is responsible for the modification of ras-related proteins by geranylgeranyl group transfer [14]. Sub-

Abbreviation used: FPP, farnesyl pyrophosphate.
strate specificity of the different transferases may be determined by their β-subunits and specific sequence motifs present in the C-terminal domains of their target proteins [14–16]. These findings provide some indication that drugs able to inhibit selectively farnesyltransferase might be found. Given the potential therapeutic value of such inhibitors for the treatment of ras-dependent cancers, we have investigated the properties of p21
superscript
farnesyltransferase(s) derived from a human tissue, placenta.

Results and discussion

Extraction and properties of human placental farnesyltransferase

Human placenta was found to be a convenient source for the preparation of farnesyltransferase activity. Farnesyltransferase was detected by measuring the incorporation of [3H]farnesyl from [3H]farnesyl pyrophosphate [FPP] into recombinant p21
superscript
farnesylated synthesized by Escherichia coli, as described in the legend to Table 1. Enzyme activity was found predominantly (93% of total) in the soluble fraction prepared from a placental homogenate. Partial purification (60-fold) of the transferase activity was achieved by ammonium sulphate fractionation and ion-exchange chromatography, with about 73% recovery of activity. One major peak (eluting at ~0.35 M-NaCl) and several minor peaks of farnesyltransferase activity were resolved by chromatography on Q-Sepharose, suggesting that some molecular heterogeneity might exist. The major peak, which was used for further studies, contained a form of farnesyltransferase which migrated as a single species during gel permeation chromatography (G200 Superdex HR), with an apparent relative molecular mass of 112000. The chromatographic properties of this human farnesyltransferase therefore, more closely resemble those reported for the enzyme from rat brain (M, 70000–100000) than larger forms reported in porcine kidney (M, 250000–300000) and bovine brain (M, 190000).

We have yet to determine whether the placental enzyme exists as a heterodimeric complex of α and β subunits structurally related to that described for the rat brain enzyme. Determination of apparent K,
subscripts
values for the two enzyme substrates [1H]FPP and p21
superscript
farnesylated, gave values of 63 nM and approximately 5 μM respectively.

Substrate specificity

The partially purified placental transferase was not specific for p21
superscript
farnesylated but also farnesylated p21
superscript
farnesylated. However, the enzyme had no activity towards a mutant form of p21
superscript
farnesylated (V12, S166) in which the normal farnesylation site CVLS has been changed to SVLS. Since this mutant retains two cysteine residues upstream from the farnesylated site, which are normally subject to modification by palmitoylation [6], this result provides evidence that the farnesyltransferase is able to retain a high degree of

| Table 1 |

Purification of human placental farnesyltransferase

Farnesyltransferase activity was purified from the supernatant fraction (10000 g) of a placental homogenate essentially as described in [9]. The 30–50% ammonium sulphate (AS) fraction was applied to a column of Q-Sepharose (40 × 3 cm) equilibrated in 50 mM-TrisCl, 50 mM-NaCl, 100 μM-ZnCl2, and 1 mM-dithiothreitol, pH 7.5 at 4°C. Column elution was carried out using a linear gradient of NaCl (0.05–1.0 M) in loading buffer. Farnesyltransferase activity in the fractions was assayed in a final volume of 25 μL containing 50 mM-TrisCl, 5 mM-MgCl2, 50 μM-ZnCl2, 5 mM-dithiothreitol, 1 mg/ml trypsin inhibitor, 5 μM p21
superscript
farnesylated and 0.33 μM [3H]FPP. Reactions were carried out in wells of a microtiter plate for 60 min at 37°C and were terminated by the addition of 100 μL of 12.5% (v/v) trichloroacetic acid containing 1% SDS. After precipitation overnight at 4°C reaction products were filtered and counted on a Beta Plate scintillation counter.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (pmol/h)</th>
<th>Specific activity (pmol h⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant (10000 g)</td>
<td>450</td>
<td>16 965</td>
<td>311 400</td>
<td>18.4</td>
</tr>
<tr>
<td>AS (0–30%)</td>
<td>68</td>
<td>700</td>
<td>26 180</td>
<td>37.4</td>
</tr>
<tr>
<td>AS (30–50%)</td>
<td>185</td>
<td>3 090</td>
<td>2 749 10</td>
<td>89.0</td>
</tr>
<tr>
<td>Q-Sepharose-1</td>
<td>160</td>
<td>2 16</td>
<td>2 276 80</td>
<td>105 40</td>
</tr>
<tr>
<td>Q-Sepharose-2</td>
<td>141</td>
<td>1 47</td>
<td>34 404</td>
<td>2 340</td>
</tr>
</tbody>
</table>

1992
specificity in vitro. Interestingly, inclusion of the mutant protein \(p21^{K-ras}\) (V12, S186) at concentrations of up to 60 \(\mu M\) in reactions with either normal \(p21^{N-ras}\) (5 \(\mu M\)) or \(p21^{K-ras}\) (4 \(\mu M\)) did not significantly inhibit the farnesylation of these proteins. These results suggest that only the extreme C-terminal portion of the \(p21^{N-ras}\) protein, containing the cysteine farnesylation site, is important for critical interactions with the farnesyltransferase. A similar conclusion has come from published work showing that rat brain farnesyltransferase can bind to a hexapeptide containing the consensus Cys-CAAX sequence, and that corresponding tetratetrapeptides can compete with \(p21^{N-ras}\) for farnesylation by this enzyme [9].

**Inhibition of farnesyl transferase**

In view of the importance of the C-terminal CAAX domain of ras proteins for transferase recognition, we looked at the inhibitory activity of cysteine-containing peptides. Peptides derived from \(p21^{N-ras}\) or \(p21^{K-ras}\) were inhibitory with \(IC_{50}\) values of approximately 2 \(\mu M\) (Table 2). Three non-Ras related peptides containing a CAAX sequence were almost as effective, while a fourth ending with the sequence CGKN was only weakly inhibitory. Our results suggest that placental farnesyltransferase recognizes either neutral or basic residues at the penultimate position of the CAAX site, but that a basic amino acid at the C-terminus (X) is not accepted. The lack of inhibition by a peptide containing three cysteine residues out of the consensus sequence, further points to a strong positional requirement of this residue for enzyme recognition.

In conclusion, the properties of a predominant form of farnesyltransferase present in human placenta closely resemble those described for an enzyme purified from rat brain. Placental transferase recognition of the \(p21^{N-ras}\) substrate is very specifically targeted on the C-terminal cysteine residue of this protein. Consequently, inhibitors based on this CAAX recognition domain may provide a starting point for the development of anti-ras drugs with uses in cancer chemotherapy.

We gratefully acknowledge the contribution of Dr. A. Hall who generously provided the \(p21^{N-ras}\) expression plasmid.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>(IC_{50}) ((\mu M))</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Ras</td>
<td>(a.a.)-Cys-Val-Val-Met</td>
<td>2.0</td>
</tr>
<tr>
<td>K-Ras</td>
<td>(a.a.)-Cys-Val-Ile-Met</td>
<td>2.4</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>(a.a.)-Cys-Pro-Leu-Gly</td>
<td>4.2</td>
</tr>
<tr>
<td>Arg-VP</td>
<td>(a.a.)-Cys-Pro-Arg-Gly</td>
<td>3.8</td>
</tr>
<tr>
<td>Lys-VP</td>
<td>(a.a.)-Cys-Pro-Lys-Gly</td>
<td>3.2</td>
</tr>
<tr>
<td>MCDP</td>
<td>(a.a.)-Cys-Gly-Lys-Asn</td>
<td>&gt;100</td>
</tr>
<tr>
<td>MT-Peptide</td>
<td>Lys-Cys-Thr-Cys-Cys-Ala</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Lipid modifications and function of the ras superfamily of proteins

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Introduction

The ras superfamily of proteins consists of over 30 members which are all low-molecular-mass (20–30 kDa) GTP-binding proteins [1]. These can be divided on the basis of overall sequence homology into three subfamilies: ras-related, involved in differentation and growth control; rho-related, some members regulating the cytoskeleton; rab-related, controlling intracellular membrane trafficking. All of these proteins function via a cycle of GTP binding and hydrolysis. In the GDP-bound state they are in an 'inactive' conformation. Under the influence of a variety of nucleotide exchange factors, the GDP is exchanged for GTP resulting in a conformational switch to the 'active' state, in which form the protein can effect its function. The protein is turned off by hydrolysis of the GTP to GDP, often under the influence of stimulatory proteins such as ras-GAP and NF-1. Although the roles of the ras-superfamily members are diverse, most of them must be associated with the cytoplasmic face of specific cellular membranes in order to function. Targeting to these specialized sites is achieved by cooperation between at least two distinct regions of the proteins. The first is the extreme C-terminus where a series of lipid modifications increases the hydrophobicity of the proteins and thus their membrane binding affinity. Secondly, a region of protein sequence just upstream of the C-terminus (the hypervariable domain) apparently encodes the determinants of targeting to the correct destination, presumably through interaction with membrane lipid head groups and/or membrane proteins.

ras proteins

Palmitoylation of one or more cysteine residues in N- and H-ras proteins had been known for several years, but it was not until 1989 that the full processing pathway was elucidated. The key to this was the recognition that the C-terminal 'CAAX box' (C, cysteine; A, aliphatic amino acid; X, any amino acid) was the site of a series of modifications similar to those occurring on a number of secreted fungal mating factors [2–4]. These involve (i) prenylation of the cysteine with the C15 steroid precursor farnesol in isomer linkage; (ii) proteolytic removal of AAX; (iii) carboxyl-methylation of the α-carboxyl group of the cysteine residue. In the case of the N-, H- and K(A)-ras proteins these modifications are followed by palmitoylation of one or two nearby upstream cysteine residues in the hypervariable domain. In contrast, the K(B)-ras protein lacks palmitoylation sites but instead has a polybasic region which co-operates with the CAAX modifications to specify plasma membrane binding. Removal of the palmitoylation sites or the polybasic region results in normally CAAX-modified proteins which are nevertheless essentially cytosolic [5]. Interestingly, the palmitate moieties of the ras proteins turn over rapidly in vitro (t1/2 ≈ 20 min) suggesting a possible mechanism for regulation of activity by modulation of membrane binding [6].

All of the CAAX modifications can be reconstituted in vitro [7]. Farnesylation occurs in an unsupplemented reticulocyte lysate, demonstrating the presence of soluble prenylphosphate synthetases and protein: farnesyltransferase. Proteolysis and methylation, however, occur only after the addition of microsomal membranes and S-adenosylmethionine. All three of the modifications are required for full manifestation of membrane-binding activity, and this is specific since it occurs primarily to added plasma membrane-enriched fractions rather than to the endoplasmic reticulum-enriched microsomal membranes used as a source of protease and methyltransferase. Recently it has become clear that two types of CAAX box exist.