Expression of polyisoprenylated Ras proteins in the insect/baculovirus system


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Ras proteins and many related proteins possess the C-terminal motif Cys-AAX, where A is an aliphatic amino acid and X is any amino acid. This sequence directs a prenyltransferase, farnesyltransferase in the case of Ras and geranylgeranylation in most other Ras-related proteins, to prenylate the cysteine residue [1-6]. The nature of the amino acid residue X appears to determine whether the protein is a substrate for farnesylation or geranylgeranylation [5]. A protease, the properties of which have not been clearly defined, removes the three C-terminal amino acids leaving the prenylated cysteine at the C-terminus. The free carboxyl group is then carboxylmethylated by a carboxylmethyltransferase, though it is not known whether this is fully stoichiometric [7, 8]. The Ras family of proteins can be divided into two sub-groups depending upon the nature of sequences N-terminal of the Cys-AAX motif [1]. In one class, exemplified by Kirsten-Ras (Ki-Ras) (4B) and Rap1a, a polybasic region with clusters of Lys/Arg residues is present. In these there are no further modifications occur. In the other class, typified by Harvey-Ras (Ha-Ras) and N-Ras, further cysteine residues are present. These are sites for palmitoylation. These modifications, in particular the isoprenylation, are critical to the biological activity of the protein [1-4, 9, 10].

Large quantities of high quality soluble recombinant Ras proteins can be produced in prokaryotic expression systems. However, the protein produced is not modified by lipids. In contrast, the insect/baculovirus expression system carries out a wide range of post-translational modifications typical of eukaryotic cells [11, 12]. An additional advantage of this system against expression in mammalian cells is that genes whose products are toxic to mammalian cells can often be expressed. For example, Rap1a can be expressed although it was toxic in NIH3T3 cells [4]. Ha-Ras [4, 13, 14], Ki-Ras [15], Rap1a [4, 16] and Rap1b [17] have all been expressed in the insect/baculovirus system and have been shown to be processed, in some cases indistinguishably from the product seen in mammalian cells.

We have expressed c-Ha-ras and Ki-ras (4B) val-12 cDNA in the insect/baculovirus system under the control of the polyhedrin promoter [13-15]. The design and optimization of such a system has been reviewed [11, 12] and will not be described further here. With both Ha-ras and Ki-ras, the expressed protein was found to be in two forms, a membrane-associated processed form and a cytoplasmic unprocessed form. The level of expression of processed protein (approx. 1% of total cell protein) was similar for both proteins. However, the level of unprocessed protein was much higher for Ha-Ras (approx. 20% of total cell protein) than for Ki-Ras (approx. 2% of total cell protein). This suggests that with an increased level of expression a saturation of the processing machinery was occurring. A similar result was reported for Ha-Ras and Rap1A by Buss et al. [4]. Time-courses of expression suggested that during the early stages the protein was fully processed, whereas with longer times after infection an increasing proportion of the protein was unprocessed. Since the virus causes most host protein synthesis to cease, it is likely that after infection there is a decrease in the activity of the processing enzymes, so that late after infection there is insufficient enzyme activity to process all the protein.

The viral inhibition of host protein synthesis results in very clear metabolic radiolabelling

Received 6 January 1992

Abbreviations used: Ras protein, protein product of the ras gene; Ki-Ras, Kirsten-ras gene product; Ha-Ras, Harvey-ras gene product; GD1, GDP-dissociation stimulator; GD1A, GDP-dissociation inhibitor.

patterns of the recombinant ras proteins (see Fig. 1 and [13–15]). Thus, incorporation of mevalonate into Hα-Ras and Ki-Ras, and incorporation of palmitate into Hα-Ras, but not into Ki-Ras, can be readily seen with short autoradiograph exposures using whole cell extracts. The carboxymethylation of both Ki- and Hα-ras proteins was also readily demonstrated [14, 15].

Although metabolic radiolabelling demonstrated that mevalonate was incorporated into the processed ras proteins, the chemical nature of the modification was not directly established. We have since utilized electrospray mass spectrometry [11] to analyse the molecular mass of the purified processed ras proteins.

Electrospray ionization of proteins results in molecular species carrying multiple charges. In the positive mode, for proteins of average amino acid composition, about one charge is formed for every 10 residues. This brings the mass/charge ratio down to values which fall in the range of standard quadrupole mass analysers. The spectra produced are a series of multiply charged protonated molecular ions, with adjacent peaks representing species differing in charge by 1 unit. Thus, the spectrum can be used to obtain several estimates of the molecular mass, increasing the accuracy of the determination. An accuracy of better than 0.01% can be obtained with proteins of Mr 20,000–30,000. This is sufficient to uniquely define many types of post-translational modification. Simple mixtures can also be analysed and the masses of the individual components calculated. This is only quantitative if all species are equally soluble and ionizable. In practice, the solubility of the sample can often be a limiting factor. Thus, the processed Hα-ras protein, which is palmitoylated and therefore highly lipophilic, usually gave very poor spectra. However, we have obtained useful spectra of the slightly more soluble processed Ki-ras protein (Fig. 2). As a test of the technique we also obtained spectra of full-length Escherichia coli-expressed Ki-ras protein and of a recombinant protein engineered to contain only the first 166 amino acids [15]. The results are summarized in Table 1. Using baculovirus-expressed, processed, Ki-Ras the spectrum was weak and only indicated the existence of a farnesylated protein lacking the three terminal amino acids of the CysAAX motif, but not otherwise modified. Later spectra in which the protein was solubilized in formic acid, before introduction into the source, revealed the existence of a methylated form of the above protein. Presumably this modification occurred at the C-terminal carboxyl group. No peaks corresponding to geranylgeranylated protein

**Fig. 1**

Metabolic radiolabelling of recombinant ras proteins

Spodoptera frugiperda insect walls were infected with wild-type (lanes 1 & 4), or recombinant baculoviruses expressing c-Ha-ras (lanes 3 & 6) or Ki-ras(valine-12) (lanes 2 & 5) genes. Cells were labelled with either [3H]palmitate (PAL) (lanes 1–3) or with [3H]mevalonate (MVA) (lanes 4–6) and analysed by SDS/PAGE 72 h post-infection. Labelling was performed as described [11, 13–15]. The gel was analysed by fluorography. Figure adapted from [11].

**Fig. 2**

Electrospray mass spectrometry of Ki-ras proteins

Electrospray mass spectrometric analysis of Ki-ras (4B) valine 12 proteins was performed as in Table 1. The upper spectrum shows E. coli-expressed full-length, unprocessed protein. The lower spectrum shows baculovirus-expressed, farnesylated protein lacking the three C-terminal amino acids, Val, Ile and Met. The numbers above the peaks refer to the number of positive charges on each molecular species.
were seen, but absence of a signal cannot be used to prove that it was not present.

As mentioned above, the insect/baculovirus system has been used by other groups to prepare lipid-modified proteins. Rap1A has been shown to be carboxymethylated, geranylgeranylated (incorrectly stated to be farnesylated in [16]), but not palmitoylated [4]. Buss et al. [4] further showed by chemical degradation that processed baculovirus-expressed Ha-Ras was nearly exclusively farnesylated with only a small proportion being geranylgeranylated. In both cases the situation was similar to that reported in mammalian cells. Wineger et al. [17] showed that radiolabelled mevalonate was incorporated into baculovirus-expressed Rap1b but the nature of the modification was not reported. Kloc et al. [18] have also demonstrated that a Xenopus protein containing a CysAAX motif, which appears to be isoprenylated and palmitoylated in Xenopus cells, is similarly modified when expressed by baculovirus.

The significance of Ras processing has been highlighted recently in two ways. First, inhibition of the processing events might be a basis for the discovery of novel anti-cancer chemotherapeutic agents. This has been discussed by Gibbs [19]. Secondly, Takai and coworkers have demonstrated that GDP-dissociation stimulator (GDS) and GDP-dissociation inhibitor (GDI) interact specifically with processed forms of Ras-related proteins, and that the protein termed as smg p21 GDS interacts with the processed form of Ki-ras but not that of Ha-Ras [20]. The functions of GDS and GDI are not clearly understood but these proteins might be of importance in the translocation of ras family proteins from the membrane to the cytoplasm. The availability of authentically processed Ras-related proteins will be of use in studying the function of GDS, GDI and similar proteins. Furthermore, it is hoped that in the near future reconstitution of a Ras signalling pathway in vitro might be possible and for this purpose purified prenylated ras proteins will be required.

We are grateful to Drs C. J. Marshall, J. E. Hancock, A. Aitken and A. I. Magee for discussions and information and to B. N. Green and T. Hutton at VG Biotech Ltd for running the mass spectra.


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**Table 1**

Molecular masses of recombinant Ki-ras proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Measured mass</th>
<th>Expected mass</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli expressed:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full-length unprocessed</td>
<td>21468 ± 1 (12)</td>
<td>21467</td>
</tr>
<tr>
<td>Truncated (1-166)</td>
<td>18900 ± 2 (8)</td>
<td>18902</td>
</tr>
<tr>
<td><strong>Baculovirus expressed:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farnesylated, methylated and 'AAX'ed</td>
<td>21340 ± 7 (7)</td>
<td>21342</td>
</tr>
<tr>
<td>Farnesylated and 'AAX'ed</td>
<td>21328 ± 6 (9)</td>
<td>21328</td>
</tr>
</tbody>
</table>
Many proteins in eukaryotic cells contain covalently bound prenyl groups composed of repeating C, isoprenes. Two prenyl groups have been identified: farnesyl (C, geranylgeranyltransferase that recognizes proteins that terminate in the sequence CaaX, where C is cysteine, X is leucine, serine, glutamine or cysteine [6]. The second enzyme is a protein geranylgeranyltransferase that recognizes proteins in which the X of the CaaX sequence is leucine [7-10]. The other enzymes, also protein geranylgeranyltransferases, recognize proteins that terminate either in Cys-Ala-Cys [11] or Gly-Gly-Cys-Cys [8].

The only one of these enzymes that has been purified is the protein farnesyltransferase from rat brain. The enzyme is a heterodimer composed of one α-subunit (49 kDa) and one β-subunit (46 kDa), both of which are required for catalytic activity [4, 7, 12, 13]. Sequencing of full-length cDNAs for both subunits revealed that the α and β subunits are the mammalian homologues of the RAM2 [13, 14] and the DPRI/RAMI [12, 15] gene products, respectively, of yeast. Mutations in either of these genes markedly reduces farnesyltransferase activity in yeast [16, 17].

The rat brain farnesyltransferase recognizes peptide substrates as short as four residues in length, a property that aided its purification by peptide affinity chromatography [4]. The peptide-binding function has been assigned to the β-subunit on the basis of chemical crosslinking studies [18]. The function of the α-subunit is unassigned, but it is postulated to play a role in the binding of farnesyl pyrophosphate (FPP). The holoenzyme forms a stable carrier complex with FPP that can be isolated by gel filtration [18]. Upon addition of a p21 protein, the enzyme immediately transfers its bound farnesyl group to p21. The farnesyltransferase shows a requirement for divalent cations, Mg²⁺ and Zn²⁺. Zn²⁺ is tightly enzyme bound and can be removed by dialysis against EDTA. It is essential for binding of the peptide substrate, and therefore it is probably attached to the β-subunit. Transfer of the enzyme-bound farnesyl group to the bound peptide acceptor requires Mg²⁺ [19].

The geranylgeranyltransferase that recognizes leucine-terminated CaaX proteins possesses an α-subunit that is so far indistinguishable from the α-subunit of the farnesyltransferase [7]. Both α-subunits migrate identically on SDS/PAGE gels.

Received 18 December 1991

p21αα Farnesyltransferase: purification and properties of the enzyme

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