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Post-translational processing of p21^{ras} is two-step and involves carboxyl-methylation and carboxy-terminal proteolysis

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We have studied the post-translational processing of p21^{ras} proteins. The primary translation product pro-p21 is cytosolic and is rapidly converted to a cytosolic form (c-p21) of higher mobility on SDS-PAGE. c-p21 is converted in turn to the membrane-bound mature palmitoylated form (m-p21) of slightly higher mobility. These processing steps are accompanied by increases in isoelectric point and in hydrophobicity as judged by Triton X-114 partitioning. Although the increases in electrophoretic mobility and hydrophobicity precede acylation we show that mutation of Cys186, which has been shown to block acylation, also abolishes the pro-p21 to c-p21 conversion. Thus the Cys186 residue is involved in the processing steps prior to acylation. We have identified two processing events which contribute to the pro-p21 conversion. Site-directed mutagenesis to insert tryptophan, which is not present in the wild type, followed by metabolic labelling with [^{3}H]tryptophan has allowed us to map a proteolytic processing event which removes the three C-terminal residues. In addition, both the c-p21 and m-p21 forms are carboxyl-methylated. Approximately one methyl group is incorporated per molecule of p21 at steady state, which can partially account for the increase in isoelectric point. Unlike palmitate, methyl group turnover is not observed.

Key words: acylation/methylation/p21^{ras}/processing/proteolysis

Introduction

The products of the mammalian *ras* genes and their yeast counterparts are membrane-associated, guanine nucleotide-binding proteins whose activity is regulated by GTPase action. Point mutations at certain critical sites lead to the mammalian proteins functioning as transforming proteins (Barbacid, 1987). The transforming ability of these proteins correlates with a low GTPase activity which cannot be stimulated by the GTPase activator protein, GAP (Trahey and McCormick, 1987; Gibbs *et al.*, 1988). Yeast RAS2 protein has been shown to be directly involved in activation of adenylate cyclase (Toda *et al.*, 1985), whereas the function of mammalian *ras* proteins is still obscure, although evidence exists for a role in signal transduction pathways (Wakelam *et al.*, 1986; Fleischman *et al.*, 1986; Wolfman and Macara, 1987).

In mammalian cells membrane-association of *ras* proteins is essential for their function (Willumsen *et al.*, 1984), although this requirement can be partially overcome in yeast by overexpression (Deschenes and Broach, 1987). This implies that *ras* proteins require membrane-binding to interact efficiently with other components of the signal-transducing machinery. The primary sequence of *ras* proteins does not contain any obvious membrane-spanning regions and the tight detergent-sensitive membrane binding (Grand *et al.*, 1987) is believed to be due to the post-translational attachment of palmitic acid in a thioester linkage to Cys186 near the C-terminus (Sefton *et al.*, 1982; Willumsen *et al.*, 1984; Chen *et al.*, 1985). Indeed it has been shown that removal of palmitate by mild hydroxylamine treatment can release p21$^{N\text{-}ras}$ from membranes (Magee *et al.*, 1987).

Multiple species of *ras* proteins, differing in SDS-PAGE mobility and isoelectric point, have been observed in a number of studies (Feuerstein and Ali, 1985; Fuhrer *et al.*, 1986; Shen *et al.*, 1987; Polonis *et al.*, 1987). In the p21^{ras} proteins of Harvey and Kirsten murine sarcoma viruses some of these forms appear to be due to phosphorylation; however, this modification cannot account for all the observed species of cellular *ras* proteins since these are not phosphorylated. It has previously been shown that *ras* proteins are first synthesized as a precursor which is rapidly chased into the mature form (Shih *et al.*, 1982). Recently Tamanoi *et al.* (1988) have shown that two precursors are involved in the processing of yeast RAS proteins and they and others have identified a gene (dpr1/RAM) which affects the first processing event (Powers *et al.*, 1986). On the basis of homologies with other acylated proteins, e.g. fungal mating factors (Kamiya *et al.*, 1979; Brake *et al.*, 1985; Akada *et al.*, 1987; Betz *et al.*, 1987) and yeast YPT1 (Molenaar *et al.*, 1988), it has been suggested that acylation may be preceded by removal of the last three amino acids, thus placing Cys186 at the C-terminus (Tamanoi *et al.*, 1988). In addition, Clarke *et al.* (1988) have recently suggested that the C-terminal amino acid is carboxyl-methylated.

To examine the post-translational modification steps of p21^{ras} we have studied the various forms for subcellular localization, SDS-PAGE mobility, isoelectric point and hydrophobicity by phase separation in Triton X-114 (Bordier, 1981). These studies have been made accessible by using cell systems that express high levels of *ras* proteins either from inducible promoters (Magee *et al.*, 1987) or by transient expression in COS-1 cells (Hancock *et al.*, 1988). We present evidence for the existence of two precursors of the final acylated form of mammalian of p21^{ras}. At least part of the difference in electrophoretic mobility between the mature form of p21^{ras} and the precursor can be shown to result from the removal of C-terminal amino acids. Changes in isoelectric point can be partially explained by loss of one carboxyl group by methylation. The acquisition of increased hydrophobicity, as judged by Triton X-114 partition, precedes palmitoylation.

Results

Fractionation and characterization of three forms of p21^{ras}

The T15 cell line over-expresses normal p21$^{N\text{-}ras}$ up to 50-fold under the control of a steroid-inducible MMTV-LTR promoter (McKay *et al.*, 1986). Distribution and characteristics of the different forms of p21^{ras} were studied by pulse-chase labelling, subcellular fractionation, Triton X-114 partition and isoelectric focusing (IEF; see Materials and methods). Figure 1 (upper panel) shows the SDS−PAGE analysis of immunoprecipitates of [^{35}S]methionine-labelled p21$^{N\text{-}ras}$ (lanes 1−6, 8−11), and [^{3}H]palmitate-labelled p21$^{N\text{-}ras}$ (lane 7). After a 10 min pulse the pro-p21 (M_r ~23 kd) and c-p21 (c, cytosolic; M_r ~21.5 kd) forms are the only labelled bands (lane 3); pro-p21 exactly co-migrates with recombinant p21$^{N\text{-}ras}$ (data not shown). When we fractionated the cells into S100 and P100 these two forms of p21$^{N\text{-}ras}$ were only in the S100 fraction (lanes 2 and 6). However, Triton X-114 partition separated the pro-p21 into the detergent-depleted aqueous phase (lane 11) and the c-p21 into the detergent-rich phase (lane 10). This shows that c-p21, although still cytosolic, has increased hydrophobic properties compared with pro-p21. After 1 h of chase the samples were analysed in the same way as above. The pro-p21 is lost (lanes 4 and 9), but some c-p21 protein is still present in the S100 fraction (lane 1) and separated in the detergent phase (lane 8), although it is difficult to resolve the c-p21 form from the fully mature m-p21 (m, membrane). The m-p21 acylated form (M_r ~21 kd) is present after 1 h chase (lane 4) exclusively in the P100 fraction (lanes 1 and 5) and fractionates into the detergent phase with Triton X-114 (lane 8). The mobility of this m-p21 was identical with [^{3}H]palmitate-labelled p21$^{N\text{-}ras}$ (lane 7); [^{3}H]palmitate is only detected in membrane-associated p21$^{N\text{-}ras}$ (Magee *et al.*, 1987).

IEF of the same samples (Figure 1, lower panel) showed that each one of these three forms of p21$^{N\text{-}ras}$ has a different pI, which were calculated for pro-p21 as 5.2, for c-p21 as 5.35 and for m-p21 as 5.45. This charge difference between them suggests that the protein is modified in some way in two distinct steps.

Mutation of Cys186 to Ser186 abolishes the first step in processing

Previous studies have identified Cys186 as a potential palmitoylation site on p21^{ras} proteins since mutation of this residue to serine blocks acylation and membrane association (Willumsen *et al.*, 1984). In order to obviate the need to isolate stable cell lines expressing *ras* proteins mutated at sites involved in processing we have used transient expression of *ras* containing plasmids in COS-1 cells.

Figure 2 shows the results of an analysis of processing in COS-1 cells transfected with plasmids encoding H-Ser186 or a wild-type H-*ras* gene (KT). The wild-type pro-p21 seen in a pulse-label is found in the aqueous phase of a Triton X-114 partition system. This is chased into a faster moving mature form which partitions in the detergent phase, indicating increased hydrophobicity as was found in the T15^{+} cells. The mature p21 is membrane-bound and acylated (data not shown). Replacement of Cys186 with Ser completely abolishes the mobility shift and increase in hydrophobicity of the protein. In this experiment there is a small degree of carry-over of pro-p21 from the aqueous to the detergent phase in both the KT wild type and KT-Ser186 partitions. The KT-Ser186 mutant remains in the cytosol and as previously reported is unacylated (Willumsen *et al.*, 1984). Thus removal of Cys186 not only prevents acylation but also abolishes a processing event which precedes acylation.

p21$^{H\text{-}ras}$ undergoes proteolysis during processing

One possible explanation of the increased SDS−PAGE mobility of c-p21 compared with pro-p21 is that amino acids are removed by proteolysis. In order to address whether *ras* processing involves C-terminal proteolysis we have used site-directed mutagenesis to introduce tryptophan residues at various sites within the C-terminus. Since wild-type p21$^{H\text{-}ras}$ contains no tryptophan residues, replacement of designated amino acids with tryptophan followed by metabolic labelling with [^{3}H]tryptophan and examination of whether label is present in both the faster migrating forms and the pro-p21, allows the site of potential cleavage to be mapped. Two substitutions, Phe28 → Trp28 and Ser189 → Trp189, were made in p21$^{H\text{-}ras}$. These mutant cDNAs, 28W and 189W respectively, were subcloned into the

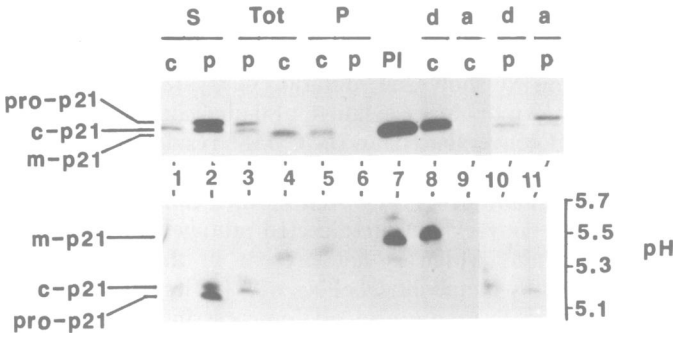

Fig. 1. SDS−PAGE and IEF of different forms of p21^{ras}. T15^{+} cells in 60 mm dishes were pulse-labelled for 10 min (p) with 100 μCi/ml [^{35}S]methionine in methionine-free medium and chased for 1 h (c) in medium containing the normal methionine concentration (**lanes 1−6, 8−11**). [^{3}H]Palmitate (100 μCi/ml) labelling (P1, **lane 7**) was done in FA medium for 2 h (Magee *et al.*, 1987). The cells were lysed in RIPA buffer (Tot); or fractionated into S100 (S) and P100 (P) as described in Materials and methods and partitioned in Triton X-114 (detergent phase = d; aqueous phase = a). All the samples were immunoprecipitated with Y13-259 monoclonal antibody, half of each sample was analysed by SDS−PAGE (upper panel) and the other half by IEF (lower panel) followed by fluorography. The result of a single experiment is shown; similar results were obtained on at least three other occasions.

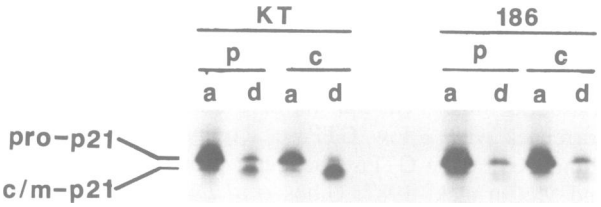

Fig. 2. Processing of p21$^{H\text{-}ras}$ is blocked in a Ser186 mutant. COS-1 cells transfected with Val12 H-*ras* (KT) or KT-Ser186 (186) were pulse-labelled (p) with [^{35}S]methionine for 15 min and chased (c) for 4 h. Lysates were made in Triton X-114 and partitioned into aqueous (a) and detergent (d) phases followed by immunoprecipitation as described in Materials and methods. The processed cytosolic (c-) and membrane (m-) forms are not resolved in this experiment.

SV40-based expression vector pEXV-3 (Miller and Germain, 1986). Table I shows that both of these substituted proteins retain transforming activity assayed by focus formation on NIH 3T3 cells. To examine the biosynthesis of these p21^{ras} proteins COS-1 cells were transiently transfected with EXV-*ras* plasmids, metabolically labelled for 12 h with [^{3}H]tryptophan and the proteins analysed by immunoprecipitation. Panel A of Figure 3 shows immunoprecipitates of COS-1 cells expressing wild-type or Trp-substituted p21; two bands are visible in lane d corresponding to the pro and processed forms of the 28W substituted protein. However, for the 198W substituted protein only a single slower-migrating band contains [^{3}H]tryptophan (lane e), indicating that the tryptophan residue at position 189 is present only in the pro-form of the 189W mutant protein. As expected no [^{3}H]tryptophan label is incorporated into the wild-type protein in control lane c.

To confirm that the 189W substituted protein is processed in the same manner as the wild-type protein, COS cell transients were pulse-labelled with [^{35}S]methionine for 15 min and either lysed immediately in 1% Triton X-114 or chased for 4 h before lysis. The lysates were phase separated, immunoprecipitated and analysed by SDS–PAGE

Table I. Transforming efficiency of H-ras tryptophan substitutions

Construct	Foci/μg	Relative activity
EXV-H (Gly12)	100	1.0
EXV-H 28W (Gly12, Trp28)	84	0.84
EXV-H 189W (Gly12, Trp189)	25	0.25

Focus assays on NIH 3T3 cells were carried out as previously described (Marshall *et al.*, 1982). Varying amounts of pEXV plasmid (1–1000 ng/plate) were precipitated with calcium phosphate in the presence of 20 μg/plate normal human DNA. The number of morphologically transformed foci was determined 13-15 days after transfection. The transforming efficiencies of these constructs are expressed relative to wild-type (Gly12) H-ras. The 28W and 189W substituted H-ras genes do not have an activating mutation.

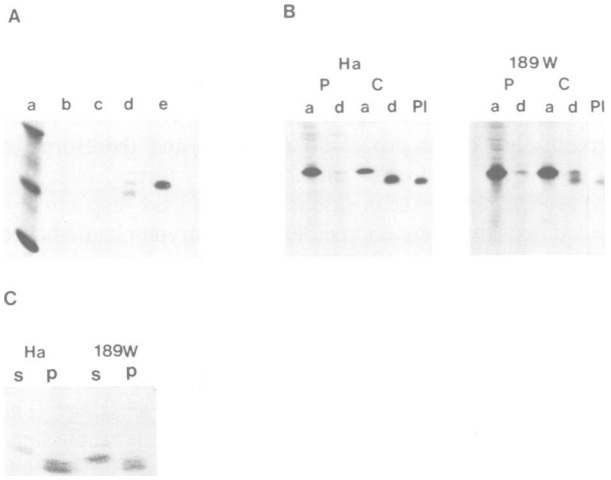

Fig. 3. (**A**) [^{3}H]Tryptophan labelling of p21$^{H\text{-}ras}$ with tryptophan substitutions. Sixty-hour post-transfection COS cells were labelled for 12 h with 200 μCi/ml L-[5-^{3}H]tryptophan. Cells were extracted with 1% Triton X-100, 0.5% sodium deoxycholate, 100 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris–HCl, pH 7.4. 2 × 10^7 TCA-precipitable c.p.m. were immunoprecipitated, resolved by SDS–PAGE on a 15% gel and visualized by fluorography for 2 days. (**a**) Mol. wt markers (30 kd, 18 kd and 14 kd); (**b**) 28W mutant p21$^{H\text{-}ras}$ (no antibody control); (**c**) wild-type p21$^{H\text{-}ras}$; (**d**) 28W mutant p21$^{H\text{-}ras}$; (**e**) 189W mutant p21$^{H\text{-}ras}$. (**B**) Processing of 189W mutant and wild-type *ras* proteins. Twelve hours after transfection COS cells were reseeded to 20 mm dishes; 48 h later two dishes per transfection were switched to methionine-free medium for 1 h followed by 450 μCi/ml [^{35}S]methionine for 15 min. One plate was lysed immediately, the other was chased for a further 4 h before lysis. The third dish was labelled with 100 μCi/ml [^{3}H]palmitic acid. Lysates were prepared in 1% Triton X-114 in TBS, phase separated, immunoprecipitated and resolved as in (A). [^{3}H]Palmitic acid lysates were not partitioned. Ha, wild-type p21$^{H\text{-}ras}$; 189W, 189W mutant p21$^{H\text{-}ras}$; P, pulse; C, chase; a, aqueous phase; d, detergent phase; Pl, palmitic acid label. (**C**) Western blot of supernatant and membrane fractions of COS cell transients. 40 μg total protein from P100 and S100 fractions of COS cell transients, 60 h post-transfection, was loaded per lane and resolved by SDS–PAGE on a 15% gel. Following electrotransfer to nitrocellulose paper, immunoblotting was performed using a polyclonal rabbit anti-p21^{ras} serum and developed using alkaline phosphatase conjugated anti-rabbit antibody. S, 10- to 20-fold concentrated S100 fraction; P, P100.

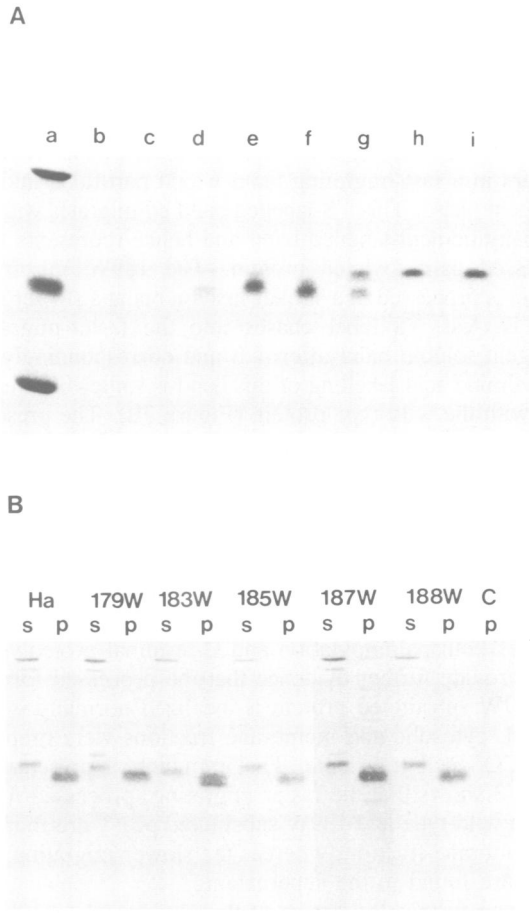

Fig. 4. (**A**) [^{3}H]Tryptophan labelling of p21$^{H\text{-}ras}$ tryptophan substituted proteins. COS cell transients were labelled for 12 h with [^{3}H]tryptophan before preparation of the lysates and immunoprecipitation as described in the legend to Figure 3. (**a**) Mol. wt markers as in Figure 3; (**b**) wild-type; (**c**) 28W mutant (no antibody control); (**d**) 28W mutant; (**e**) 179W mutant; (**f**) 183W mutant; (**g**) 185W mutant; (**h**) 187W mutant; (**i**) 188W mutant. (**B**) Western blot of supernatant and membrane fractions of COS cell transients. Blots performed as described in the legend to Figure 3. P, P100 fraction; S, 10- to 20-fold concentrated S100 fraction; C, mock transfected COS cells (controls).

Table II. Incorporation of methyl groups into $p21^{N\text{-}ras}$ forms

	c.p.m. incorporated		
	Methanol (MeOH)	Methionine (Met)	Met/MeOH ratio
1. Pulse–chase (p-c)			
pro-p21 (p)	0	380	–
c-p21 (p)	125	313	2.50
m-p21 (c)	216	896	4.15
2. 18 h label			
c/m-p21	720	4297	5.97
3. Turnover			
0 min chase	530	3603	6.80
30 min chase	482	2771	5.75
60 min chase	514	2869	5.60
120 min chase	566	3449	6.10

Note: The primary sequence of the N-*ras* contains six methionine residues. Our data show that one of these is removed in pro-p21 → c-p21 conversion. It is not known whether the initiator methionine is present in any of the forms.

alongside immunoprecipitations of duplicate transfections labelled for 4 h with [^{3}H]palmitic acid. The results of this experiment are shown in panel B of Figure 3. After a 15 min pulse label most of the [^{35}S]methionine-labelled wild-type protein is found as the slow-migrating pro-p21 form which partitions into the aqueous phase after Triton X-114 extraction. Following the 4 h chase most of the ^{35}S label appears in a fast migrating band which partitions into the detergent phase. This ^{35}S-labelled band co-migrates with the [^{3}H]palmitic acid-labelled band and hence represents fully processed palmitoylated protein. The 189W substituted protein is processed in a similar fashion but at a slower rate. That is, less ^{35}S label chases into the faster-migrating detergent-soluble band after 4 h and correspondingly the [^{3}H]palmitic acid labelling of this band is somewhat weaker than with the wild-type protein (Figure 3B). The presence of tryptophan at position 189 in $p21^{ras}$ therefore slows the rate of processing of the pro-form to the mature form which may explain why the transforming activity of the 189W mutant is retained but may be reduced (Table I). Nevertheless the mature, fast-migrating, palmitoylated form of the 189W substituted protein is not labelled with [^{3}H]tryptophan, demonstrating that the processing of the pro-form of $p21^{ras}$ includes both palmitoylation and C-terminal proteolysis.

To provide further evidence that the processed form of the 189W substituted protein is localized normally within the cell, cytosolic and membrane fractions were prepared from COS cell transients. The immunoblot presented in Figure 3 shows that the faster migrating, processed forms of both wild-type and 189W substituted $p21^{ras}$ are found in the membrane fraction, whereas the slower migrating pro-forms are found in the supernatant.

To map further the extent of the C-terminal proteolysis we have made a series of further single amino acid substitutions within the C-terminus. Using site-directed mutagenesis, residues Pro179, Ser183, Lys185, Val187 and Leu188 were replaced with tryptophan and the resulting mutant cDNAs, designated 179W, 183W, 185W, 187W and 188W respectively, were subcloned into pEXV-3. Figure 4A shows immunoprecipitates of COS-1 cells transiently expressing each of these $p21^{ras}$ proteins and metabolically labelled with [^{3}H]tryptophan for 12 h before preparation of the cell lysates. The 187W and 188W substituted proteins have identical properties to the 189W mutant in that only a single slow-migrating band (lanes h and i) is labelled with [^{3}H]tryptophan. Western blotting of supernatant and membrane fractions from duplicate transfections (Figure 4B) shows that as for the 189W protein the 187W and 188W proteins are being processed normally and therefore the faster-migrating membrane-associated forms lack the tryptophan residue at positions 187 and 188 respectively. In contrast, the immunoprecipitated [^{3}H]tryptophan-labelled 179W, 183W and 185W proteins all run as doublets (Figure 4A, lanes d–g) indicating that the substituted residues are present both in the pro- and processed forms.

These data therefore map the termination site of the m-p21 protein to Cys186, the three C-terminal amino acids having been removed from pro-p21 during processing. The processing of the 179W and 183W mutant occurs at the same rate as the wild-type protein (data not shown). The doublets in lanes e and f of Figure 4A therefore have the same distribution of label between pro and m-p21 as the control (28W, lane d). The processing of the 185W, 187W and 188W proteins, however, slowed to the same rate as the 189W protein (compare Figure 3B). Interestingly, however, in COS cells 60 h post-transfection accumulation of m-p21 is very similar for all of these mutants (Figures 3C and 4A). This probably indicates that in COS cells *ras* expression is so high that the membrane can be saturated with m-p21 even when its rate of production is slowed. Also, certain of the immunoblots resolve membrane bound p21 into a tight doublet indicating that there may be two forms of this protein. Experiments are in progress in an attempt to address this possibility.

$p21^{N\text{-}ras}$ is carboxyl-methylated during processing

One possible explanation for the increased pI of $p21^{N\text{-}ras}$ during maturation is the loss of negative charge due to carboxyl-methylation. This modification is known to occur on a number of fungal mating factors with C-terminal sequences related to *ras* proteins (Sakagami *et al.*, 1981; Ishibashi *et al.*, 1984). To test for carboxyl-methylation of $p21^{N\text{-}ras}$ we labelled T15$^+$ cells with [^{3}H-methyl]methionine in a short pulse (10 min) followed by a chase (160 min), or continuously overnight (18 h). $p21^{N\text{-}ras}$ was immunoprecipitated, separated by SDS–PAGE and the incorporation

of label as methyl groups and as intact methionine into each processing intermediate was determined (Table II). No methyl groups could be detected in pro-p21. However, both c-p21 and m-p21 incorporated methyl groups. At steady state (18 h label) the ratio of label in methyl groups to methionine was ~6, indicating approximately one methyl group per p21$^{N\text{-}ras}$ molecule assuming equal specific activity of the methionine and *S*-adenosylmethionine pools (Chelsky *et al.*, 1985).

The fatty acid moiety of p21$^{N\text{-}ras}$ has been shown to undergo rapid turnover *in vivo* ($t_{1/2}$ ~20 min; Magee *et al.*, 1987). It was of interest to see if the methyl modification also underwent turnover. The methyl ester was stable *in vivo* for up to 2 h (Table II.3) or for longer times (data not shown).

Discussion

In this paper we demonstrate that post-translational processing of mammalian p21^{ras} proceeds through two kinetically distinguishable precursors (pro-p21, c-p21) which differ in SDS–PAGE mobility, hydrophobicity and pI. The second processing event (c-p21 → m-p21) is associated with strong membrane binding. This membrane binding of p21^{ras} can be partially reversed by mild hydroxylamine treatment, which removes palmitic acid and results in the deacylated protein migrating on SDS–PAGE with the same mobility as c-p21 (Magee *et al.*, 1987).

At least part of the increase in SDS–PAGE mobility of c-p21 can be accounted for by the removal of three amino acids from the C-terminus of pro-p21. The removal of terminal amino acids is analogous to the cleavage of the last three amino acids from the C-terminus of several fungal mating factors, which also contain a Cys-A-A-X sequence (A, aliphatic; X, any amino acid) at their C-termini (Sakagami *et al.*, 1981; Ishibashi *et al.*, 1984; Brake *et al.*, 1985). Removal of amino acids may account for all of the increase in SDS–PAGE mobility, although the loss of three hydrophobic amino acids would not be expected to generate a decrease in apparent mol. wt of ~1500 kd. In addition the observed changes in isoelectric point are not readily accounted for by loss of these amino acids.

Both c-p21 and m-p21 are methyl-esterified. Assuming an equal specific activity of the methionine and *S*-adenosylmethionine pools after an overnight label, the stoichiometry of methylation is about one methyl group per p21 molecule. We have not determined which residue is methylated; it could be an Asp or Glu, or the α-carboxyl of the C-terminal amino acid produced by carboxypeptidase action. Clarke *et al.* (1988) have postulated that the C-terminal amino acid of p21^{ras} is methylated. Methylation appears to be irreversible since we have been unable to detect any turnover of methyl groups in p21^{ras}. Carboxyl-methylation would account for the increase in pI during processing of pro-p21 to c-p21 but the further increase in conversion of c-p21 to m-p21 is not readily explained. It may be due to modification of another charged residue or possibly due to a conformational change resulting in altered p*K* of charged residues.

Mutation of Cys186 to Ser has been shown to abolish acylation, membrane binding and transformation by p21^{ras} (Willumsen *et al.*, 1984). This has implicated Cys186 as the acylation site. Our results are not inconsistent with these findings, but we observe that the Ser186 mutation blocks the processing of pro-p21 to c-p21, a step which we show precedes acylation. Thus the sequence Cys-A-A-X, which has been proposed to signal acylation, may actually signal a modification prior to acylation. Possible candidates for this modification are carboxypeptidase action and methylation. Methylation appears to take place on protein that has had the C-terminal amino acids removed (Clarke *et al.*, 1988) and may be involved in the acquisition of detergent binding properties by the cytosolic c-p21.

Chen *et al.* (1985) have reported the co-migration on HPLC of the synthetic peptide Cys-Val-Leu-Ser with a hydroxylamine-treated [^{35}S]cysteine-labelled tryptic peptide of acylated p21$^{H\text{-}ras}$. This peptide also co-migrated with a [^{35}S]cysteine-labelled tryptic peptide of recombinant p21$^{H\text{-}ras}$. On the basis of these observations it was proposed that p21$^{H\text{-}ras}$ was palmitoylated on Cys186 of an intact C-terminal sequence. Our data are inconsistent with this interpretation. The discrepancy could be explained by fortuitous co-migration of the authentic processed and methylated C-terminal peptide with Cys-Val-Leu-Ser, or by the low stoichiometry of the ^{35}S-labelled peptide noted by Chen *et al.* (1985).

A parallel situation exists in yeast where the RAM/dpr1 mutation has been found to affect a modification of RAS proteins prior to acylation, resulting in aberrant localization and function (Powers *et al.*, 1986; Tamanoi *et al.*, 1988). This mutation also prevents processing and secretion of a-factor, a mating factor which contains the Cys-A-A-X signal in its gene sequence (Brake *et al.*, 1985). A number of related fungal mating factors have been found to contain a C-terminal methylated cysteine residue with a polyisoprenoid side chain in ether linkage to the thiol group (Sakagami *et al.*, 1979; Ishibashi *et al.*, 1984). The existence of the Cys-A-A-X sequence in a number of other proteins, including nuclear lamins and cyclic GMP phosphodiesterase (Clarke *et al.*, 1988; Magee and Hanley, 1988), suggests that the events proposed here may be of widespread occurrence and importance.

Many *in vitro* studies of p21^{ras} function utilize recombinant protein produced in *Escherichia coli*. We and others have shown that post-translational processing of p21^{ras} does not occur in this organism (this paper; Chen *et al.*, 1985). Since these events are essential for *ras* protein function *in vivo* (Willumsen *et al.*, 1984) it may be that crucial properties are being overlooked. It will be of prime importance in future to characterize these post-translational modifications in detail, to use fully modified *ras* proteins for *in vitro* reconstitution studies and to understand whether post-translational modifications are involved in regulating the activity of *ras* proteins.

Materials and methods

Cell culture and immunoprecipitation

Culture of T15 cells has been described (Magee *et al.*, 1987). Cells were labelled for the indicated times with [^{35}S]methionine (>1000 Ci/mmol, Amersham International, Bucks, UK), Tran[^{35}S]-label (ICN Biomedicals Ltd, Bucks, UK) or [^{3}H-methyl]methionine (85 Ci/mmol, Amersham) in medium containing dialysed calf serum and no methionine, or one-tenth of the normal concentration. Alternatively cells were labelled with L-[5-^{3}H]tryptophan (Amersham, 30 Ci/mmol) in tryptophan-free medium or with [9,10(*n*)-^{3}H]palmitic acid (Amersham, 54 Ci/mmol) in normal medium supplemented with 5 mM sodium pyruvate (FA medium). Chase medium contained the normal levels of amino acids. Cells were immunoprecipitated with monoclonal antibody Y13-259 (Furth *et al.*, 1982) as previously described (Magee *et al.*, 1987).

SDS–PAGE and isoelectric focusing (IEF)

Samples were immunoprecipitated, and half was used for SDS–PAGE as described before (Magee *et al.*, 1987). The other half was dissolved in IEF sample buffer [9.5 M urea, 2% (v/v) NP-40, 2% (v/v) ampholyte 3-10, 100 mM DTT]. IEF used 3% ampholines (Pharmalyte, Pharmacia AB, Uppsala, Sweden) pH 3–10 or pH 4–6.5 in 1 mm-thick polyacrylamide gels (4% acrylamide: 230 × 115 mm) using an LKB Multiphor apparatus. Electrophoresis was usually for 16 h at room temperature at constant voltage (400 V) and bands were detected by fluorography. The pH gradient was determined by elution of the ampholines from pieces of gel in distilled water and measuring the pH. For immunoblotting, samples were transferred to nitrocellulose paper (Hybond-C-Extra, Amersham). Blots were developed using alkaline phosphatase-conjugated anti-rabbit antibody.

Carboxyl-methylation

Confluent T15$^+$ cells were labelled overnight (18 h) with 50 μCi/ml [^{3}H-methyl]methionine or pulse-labelled (10 min) with 200 μCi/ml [^{3}H-methyl]methionine followed by a chase (160 min) with cold medium. To assay methyl group turnover cells were labelled overnight to steady state, then chased for 0, 30, 60 or 120 min with cold medium. p21$^{N\text{-}ras}$ was immunoprecipitated and separated by SDS–PAGE, followed by fluorography with salicylate (Chamberlain, 1979). After exposure of film the regions of gel corresponding to pro-p21, c-p21 and m-p21 were cut out and treated overnight with NaOH to release esterified methyl groups as described by Chelsky *et al.* (1984). The label released as methanol and the label remaining as methionine were determined by scintillation counting after neutralization of the alkali with HCl.

Subcellular fractionation

Cells were fractionated into 100 000 *g*-soluble (S100) and -insoluble (P100) fractions following hypotonic lysis as previously described (Magee *et al.*, 1987). The S100 fraction was concentrated ~ 10-fold by vacuum ultra-filtration using an Amicon immersible CX10 unit at 4°C. Protein was assayed using the Bradford reaction (Bio-Rad, Herts, UK).

Triton X-114 separation (Bordier, 1981)

After labelling the cells were washed twice in ice-cold PBS and lysed directly in 1% Triton X-114 in TBS buffer (20 mM Tris, 150 mM NaCl, pH 7.5). After 10 min on ice with occasional mixing, insoluble material was removed by centrifugation at 10 000 *g* for 10 min at 4°C. The supernatants were transferred into new tubes and warmed at 37°C in a water bath for 2 min. The turbid solution was centrifuged at 10 000 *g* for 2 min at room temperature. The upper phase was the aqueous phase (detergent-depleted) and the lower was the detergent-enriched phase. After this separation the volumes of the samples were equalized by addition of TBS or 11% Triton X-114 followed by immunoprecipitation and SDS–PAGE or IEF.

COS cell transfections and mutagenesis

COS-1 cells were transfected using a DEAE–dextran method as described previously (Hancock *et al.*, 1988). *In vitro* oligonucleotide-directed mutagenesis was performed after the methods of Taylor *et al.* (1985). All mutated cDNAs were fully sequenced before insertion into the pEXV-3 expression vector.

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References

Akada,R., Minomi,K., Yamashita,I., Miyakawa,T. and Fukui,S. (1987) *Agric. Biol. Chem.*, **51**, 1211–1215.

Barbacid,M. (1987) *Annu. Rev. Biochem.*, **56**, 779–827.

Betz,R., Crabb,J.W., Meyer,H.E., Wittig,R. and Duntze,W. (1987) *J. Biol. Chem.*, **262**, 546–548.

Bordier,C. (1981) *J. Biol. Chem.*, **256**, 1604–1607.

Brake,A.J., Brenner,C., Najarian,R., Laybourne,P. and Merryweather,J. (1985) In Gething,M.-J. (ed.), *Protein Transport and Secretion.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 103–108.

Chamberlain,J.P. (1979) *Anal. Biochem.*, **98**, 132–135.

Chelsky,D., Gutterson,N.I. and Koshland,D.E.,Jr (1984) *Anal. Biochem.*, **141**, 143–148.

Chelsky,D., Ruskin,B. and Koshland,D.E.,Jr (1985) *Biochemistry*, **24**, 6651–6658.

Chen,Z-Q., Ulsh,L.S., DuBois,G. and Shih,T.Y. (1985) *J. Virol.*, **56**, 607–612.

Clarke,S., Vogel,J.P., Deschenes,R.J. and Stock,J. (1988) *Proc. Natl. Acad. Sci. USA.*, **85**, 4643–4647.

Deschenes,R.J. and Broach,J.R. (1987) *Mol. Cell Biol.*, **7**, 2344–2351.

Feuerstein,N. and Ali,I.U. (1985) *J. Cell Biochem.*, **29**, 253–263.

Fleischman,L.F., Chahwala,S.B. and Cantley,L. (1986) *Science*, **231**, 407–410.

Furth,M.E., Davis,L.J., Fleurdelys,B. and Scolnick,E.M. (1982) *J. Virol.*, **43**, 294–304.

Fuhrer,J.P., De Biasi,F., Cooper,H.L. and Schlom,J. (1986) *Biochim. Biophys. Acta*, **866**, 204–215.

Gibbs,J.B., Schaber,M.D., Allard,W.J., Sigal,I.S. and Scolnick,E.M. (1988) *Proc. Natl. Acad. Sci. USA.*, **85**, 5026–5030.

Grand,R.J.A., Smith,K.J. and Gallimore,P.H. (1987) *Oncogene*, **1**, 305–314.

Hancock,J.F., Marshall,C.J., McKay,I.A., Gardner,S., Houslay,M.D., Hall,A. and Wakelam,M.J.O. (1988) *Oncogene*, **3**, 187–193.

Ishibashi,Y., Sakagami,Y., Isogai,A. and Suzuki,A. (1984) *Biochemistry*, **23**, 1399–1404.

Kamiya,Y., Sakurai,A., Tamura,S., Takahashi,N., Tsuchiya,E., Abe,K. and Fukui,S. (1979) *Agric. Biol. Chem.*, **43**, 363–369.

McKay,I.A., Marshall,C.J., Cales,C. and Hall,A. (1986) *EMBO J.*, **5**, 2617–2621.

Magee,A.I. and Hanley,M.R. (1988) *Nature*, **335**, 114–115.

Magee,A.I., Gutierrez,L., McKay,I.A., Marshall,C.J. and Hall,A. (1987) *EMBO J.*, **6**, 3353–3357.

Marshall,C.J., Hall,A. and Weiss,R.A. (1982) *Nature*, **299**, 171–173.

Miller,J. and Germain,R.N. (1986) *J. Exp. Med.*, **164**, 1478–1489.

Molenaar,C.M.T., Prange,R. and Gallwitz,D. (1988) *EMBO J.*, **7**, 971–976.

Polonis,V.R., Anderson,G.R. and Doyle,D. (1987) *Arch. Biochem. Biophys.*, **254**, 541–546.

Powers,S., Michaelis,S., Broek,D., Santa Anna-A.S., Field,J., Herskowitz,I. and Wigler,M. (1988) *Cell*, **47**, 413–422.

Sakagami,Y., Isogai,A., Suzuki,A., Tamura,S., Kitada,C. and Fujino,M. (1979) *Agric. Biol. Chem.*, **43**, 2643–2645.

Sakagami,Y., Yoshida,M., Isogai,A. and Suzuki,A. (1981) *Science*, **212**, 1525–1527.

Sefton,B.M., Trowbridge,I.S., Cooper,J.A. and Scolnick,E.M. (1982) *Cell*, **31**, 465–475.

Shen,W.P.V., Aldrich,T.H., Venta-Perez,G., Franza,B.R. and Furth,M.E. (1987) *Oncogene*, **1**, 157–165.

Shih,T.Y., Weeks,M.O., Gruss,P., Dhar,R., Oroszlan,S. and Scolnick,E.M. (1982) *J. Virol.*, **42**, 253–261.

Tamanoi,F., Hsueh,E.C., Goodman,L.E., Cobitz,A.R., Detrick,R.J., Brown,W.R. and Fujiyama,A. (1988) *J. Cell Biochem.*, **36**, 261–273.

Taylor,J.W., Ott,J. and Eckstein,S. (1985) *Nucleic Acids Res.*, **13**, 8764–8785.

Toda,T., Uno,I., Ishikawa,T., Powers,S., Kataoka,T., Broek,D., Cameron,S., Broach,J., Matsumoto,K. and Wigler,M. (1985) *Cell*, **40**, 27–36.

Trahey,M. and McCormick,F. (1987) *Science*, **238**, 542–545.

Wakelam,M.J.O., Davies,S.A., Houslay,M.D., McKay,I., Marshall,C.J. and Hall,A. (1986) *Nature*, **323**, 173–176.

Willumsen,B.M., Norris,K., Papageorge,A.G., Hubbert,N.L. and Lowy,D.R. (1984) *EMBO J.*, **3**, 2581–2585.

Wolfman,A. and Macara,I.G. (1987) *Nature*, **325**, 359–361.

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