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Methylation and proteolysis are essential for efficient membrane binding of prenylated p21$^{K\text{-}ras(B)}$

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Plasma membrane targeting of p21$^{K\text{-}ras(B)}$ requires a CAAX motif and a polybasic domain. The CAAX box directs a triplet of post-translational modifications: farnesylation, proteolysis of the AAX amino acids and methylesterification. These modifications are closely coupled *in vivo*. However, *in vitro* translation of mRNA in rabbit reticulocyte lysates produces p21$^{K\text{-}ras(B)}$ proteins which are arrested in processing after farnesylation. Intracellular membranes are then required both for proteolytic removal of the AAX amino acids and methylesterification of farnesylated p21$^{K\text{-}ras(B)}$. Binding of p21$^{K\text{-}ras(B)}$ to plasma membranes *in vitro* can then be shown to depend critically on AAX proteolysis and methylesterification since p21$^{K\text{-}ras(B)}$ which is farnesylated, but not methylated, binds inefficiently to membranes.

Key words: p21$^{K\text{-}ras(B)}$/*in vitro*/processing

Introduction

A number of proteins including fungal mating factors, nuclear lamins, the transducin γ-subunit and p21^{ras} all terminate in a C-terminal CAAX motif (C = cysteine, A = aliphatic, X = non aliphatic amino acid) and have been shown to undergo a triplet of post-translational modifications. This set of modifications comprises prenylation of the cysteine residue with a C_{15} farnesyl moiety, proteolysis to remove the -AAX amino acids and carboxymethylation (Wolda and Glomset, 1988; Clarke *et al.*, 1988; Gutierrez *et al.*, 1989; Hancock *et al.*, 1989; Casey *et al.*, 1989; Lai *et al.*, 1990; Fukada *et al.*, 1990). For the *Saccharomyces cerevisiae* a-type mating factor and the transducin γ-subunit, structural determination has shown that following post-translational processing: cysteine is the C-terminal amino acid, farnesyl is linked to the sulphur by a thio ether bond and the α-carboxyl group of the cysteine is methylesterified (Anderreg *et al.*, 1988; Lai *et al.*, 1990; Fukada *et al.*, 1990). Although the site of carboxylmethylation has not been determined in p21^{ras} it is generally assumed to be on the C-terminal cysteine because, in all other features, the post-translational modifications of p21^{ras} resemble the other proteins.

In the case of the nuclear lamins and p21^{ras}, the post-translational modifications of the CAAX motif combine with another signal to target localization to specific membranes (Holtz *et al.*, 1989; Hancock *et al.*, 1990). For the localization of p21^{ras} to the inner surface of the plasma membrane, the second signal is contained within the C-terminal hypervariable domain and comprises a cysteine palmitoylation site (p21$^{H\text{-}ras}$, p21$^{N\text{-}ras}$) or a polybasic sequence [p21$^{K\text{-}ras(B)}$] (Hancock *et al.*, 1990). The net result of the CAAX modifications is to significantly increase the hydrophobicity of the processed protein (Gutierrez *et al.*, 1989). However, it has not been possible to determine whether all three post-translational modifications are required for membrane binding. In order to investigate the individual contributions of the CAAX modifications to membrane association we have made use of *in vitro* translated proteins.

Previous studies have shown that CAAX containing peptides are farnesylated *in vitro* in soluble cellular extracts (Reiss *et al.*, 1990; Schafer *et al.*, 1990) and that nuclear lamins A and B can be prenylated in a rabbit reticulocyte lysate (Vorburger *et al.*, 1989). In addition an enzyme has been identified in rat liver membranes which can methylesterify synthetic farnesyl:cysteine containing peptides (Stephenson and Clarke, 1990). These peptides mimic the C-terminal structure of farnesylated, -AAX proteolysed ras proteins, and hence the enzyme is an excellent candidate for the p21^{ras} methyltransferase. Genetic studies in yeast suggest that the methyltransferase activity is encoded by, or dependent on, the STE14 gene product (Hrycyna and Clarke, 1990).

We now show that p21$^{K\text{-}ras(B)}$ is prenylated in reticulocyte lysates but that neither proteolysis nor methylation takes place. However, addition of pancreatic microsomes results in proteolysis and methylation to produce a fully processed p21$^{K\text{-}ras(B)}$ protein. Use of an inhibitor of the methylation step, in the presence of microsomes, allows prenylation and proteolysis but not methylation to occur. Thus, the two potential intermediates in processing, and the fully processed form of p21$^{K\text{-}ras(B)}$, can be assayed for binding to membranes.

Results

Post-translational processing of p21$^{K\text{-}ras(B)}$ in an in vitro system

Three K-*ras* cDNAs encoding wild-type p21$^{K\text{-}ras(B)}$, a K-ras protein with a Cys186 → Ser mutation (K186) and a K-ras protein with the polybasic domain replaced with uncharged glutamine residues (K6Q) (Hancock *et al.*, 1990) were cloned into pGEM plasmids. The genes were transcribed *in vitro* and the mRNA produced used to direct *in vitro* translation in a nuclease treated (message dependent) rabbit reticulocyte lysate. The extent of the post-translational processing of the translated proteins was then determined.

Post-translational modifications which occur at the C-terminal CAAX motif increase the hydrophobicity of p21^{ras} (Gutierrez *et al.*, 1989; Hancock *et al.*, 1989). This is readily assayed by the partitioning of radiolabelled ras proteins after Triton X-114 phase separation, followed by

immunoprecipitation and gel electrophoresis (Bordier, 1981; Gutierrez *et al.*, 1989; Hancock *et al.*, 1989). Aliquots of reticulocyte lysates containing [^{35}S]methionine labelled p21$^{K\text{-}ras(B)}$ proteins were therefore assayed by Triton X-114 partitioning. Figure 1 shows that the K186S control protein, which is blocked for step I processing (Hancock *et al.*, 1989), is found exclusively in the aqueous phase while 20% of wild-type K-ras protein partitions into detergent. This result indicates that only a small proportion of the translated protein is being processed in the unmodified lysate.

To investigate whether the low level of processing was due to an inadequate supply of mevalonic acid (MVA, the precursor of farnesyl pyrophosphate) the lysates were supplemented with MVA during the translation incubation. Under these conditions 70% of the translated wild-type protein partitioned into the detergent phase of Triton X-114. MVA supplementation had no effect on the processing of the K186 protein (Figure 1). The K6Q protein was processed to the same extent as wild-type p21$^{K\text{-}ras(B)}$ in the MVA supplemented lysate (Figure 1). We have previously shown that mutations in the polybasic domain of p21$^{K\text{-}ras(B)}$ have no detrimental effect on processing of the CAAX motif *in vivo* (Hancock *et al.*, 1990). To confirm that p21$^{K\text{-}ras(B)}$ was being prenylated *in vitro*, translations were performed in lysates supplemented with [^{14}C]mevalonolactone. Figure 2 shows that label was incorporated into both wild-type p21$^{K\text{-}ras(B)}$ and the K6Q mutant but not into the K186 protein. A recent study has shown that rabbit reticulocyte lysate farnesylates recombinant, *Escherichia coli* expressed p21^{ras} protein (Schaber *et al.*, 1990), suggesting strongly that the prenyl group attached to p21$^{K\text{-}ras(B)}$, translated *in vitro*, is C_{15} farnesyl.

Following farnesylation, p21^{ras} undergoes proteolysis to remove the -AAX amino acids (Gutierrez *et al.*, 1989) and methylesterification (Clarke *et al.*, 1989; Gutierrez *et al.*, 1989). By analogy with other CAAX containing proteins (Anderegg *et al.*, 1988) the methylesterification is assumed to occur on the newly exposed C-terminal cysteine residue. To investigate whether methylesterification was occurring in the lysate, translations of p21$^{K\text{-}ras(B)}$ were performed in the presence of *S*-adenosyl-[^{3}H]methionine (SAM) as a methyl donor. Figure 3A shows that no incorporation of label into the p21$^{K\text{-}ras(B)}$ protein was evident. However, since the p21^{ras} methyltransferase has been localized to intracellular membranes (Stephenson and Clarke, 1990; Hrycyna and Clarke, 1990), the experiment was repeated in the presence of canine pancreatic microsomal membranes. Under these conditions methylation occurred on p21$^{K\text{-}ras(B)}$ and K6Q but not on the control K186 protein (Figure 3). We next investigated whether methylation could be inhibited by methylthioadenosine (MTA), which has been shown to significantly slow the methylesterification of nuclear lamins *in vivo* (Chelsky *et al.*, 1989). Translations of p21$^{K\text{-}ras(B)}$ were therefore performed with added microsomes in the presence of 3 mM MTA, which is close to the limit of solubility of the compound (Chelsky *et al.*, 1989). Control translations labelled with [^{35}S]methionine indicated that 3 mM MTA in DMSO (0.6% final concentration) inhibited translation by 29%. However, labelling of p21$^{K\text{-}ras(B)}$ by

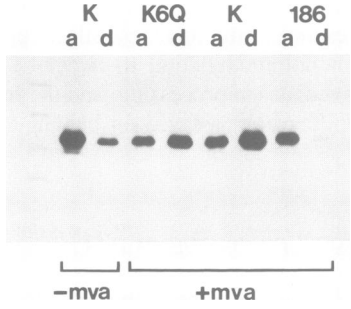

Fig. 1. Triton X-114 partitioning of *in vitro* translated K-ras proteins. K-ras proteins were translated in a reticulocyte lysate without added MVA (−mva) or with MVA at a final concentration of 5 mM (+mva). 2 μl aliquots of the [^{35}S]methionine-labelled translation reactions were partitioned in Triton X-114 and the aqueous (a) and detergent (d) phases immunoprecipitated and resolved by SDS−PAGE. To estimate the extent of processing the radioactivity incorporated into the aqueous and detergent partitioning forms of p21$^{K\text{-}ras(B)}$ was determined as described (Materials and methods). In the unmodified lysate, 20% of the translated wild-type p21$^{K\text{-}ras(B)}$ (K) is processed (i.e. partitions into detergent), whereas if the lysate is supplemented with MVA, the processed fraction increased to 70%. The K6Q mutant protein which has had the polybasic domain replaced with six glutamine residues is processed to the same extent as wild-type p21$^{K\text{-}ras(B)}$. The Cys186 → Ser mutant (186) is blocked for step I processing and partitions exclusively into the aqueous phase.

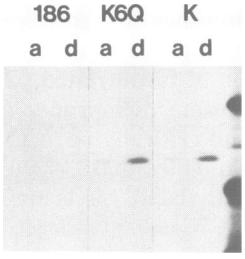

Fig. 2. Prenylation of K-ras(B) proteins *in vitro*. Translations of p21$^{K\text{-}ras(B)}$ proteins were labelled with [^{14}C]mevalonic acid lactone (in the absence of added cold MVA). 25 μl of the lysate was then partitioned in Triton X-114 and the aqueous (a) and detergent (d) phases immunoprecipitated separately. K = wild-type p21$^{K\text{-}ras(B)}$, 186 = p21$^{K\text{-}ras(B)}$ (Cys186 → Ser), K6Q = p21$^{K\text{-}ras(B)}$ [(Lys175−180) → Gln].

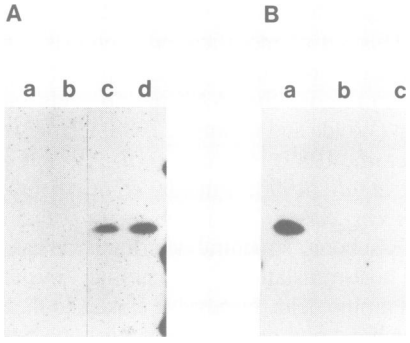

Fig. 3. Methylation of K-ras protein *in vitro*. **A**. Translations of p21$^{K\text{-}ras(B)}$ proteins were carried out in the presence and absence of canine microsomal membranes in lysates supplemented with 5 mM MVA and labelled with SAM. 25 μl lysate was immunoprecipitated, resolved by SDS−PAGE and fluorographed. a = K6Q minus microsomes, b = p21$^{K\text{-}ras(B)}$ minus microsomes, c = K6Q plus microsomes, d = p21$^{K\text{-}ras(B)}$ plus microsomes. **B**. SAM labelling of *in vitro* translated p21$^{K\text{-}ras(B)}$ (lanes a and b) and p21$^{K\text{-}ras(B)}$ Ser186 (lane c) proteins was performed in the presence of microsomes as described above, and the proteins immunoprecipitated. MTA (final concentration 3 mM) was included in the translation of p21$^{K\text{-}ras(B)}$ in lane b.

SAM was reduced to ~5% of control in the presence of 3 mM MTA (Figure 3B).

Since the methyltransferase responsible for methylating p21^{ras} is membrane bound, we wished to determine whether the protease responsible for removing the -AAX amino acids was present in the cytosol or was associated with the microsomal membranes. To address this question, tryptophan substitutions were made in the p21$^{K\text{-}ras(B)}$ protein at amino acid 28, N-terminal of the CAAX motif and amino acid 189 at the extreme C-terminus. Since there are no tryptophan residues in wild-type p21$^{K\text{-}ras(B)}$, tryptophan substitutions allow specific labelling of individual residues within the protein to determine whether a particular residue is still present in processed forms of p21^{ras} (Gutierrez *et al.*, 1989). The 28W and 189W substituted proteins were translated in [^{3}H]tryptophan supplemented lysates, with and without added microsomal membranes. Figure 4 shows that both the aqueous and detergent partitioning forms of the control 28W protein are labelled by [^{3}H]tryptophan irrespective of the presence of microsomes. However, with the 189W protein [^{3}H]tryptophan label is only found in the detergent phase in the absence of added microsomes (Figure 4B). The control ^{35}S-labelled experiment in Figure 4A shows that the tryptophan substitution at amino acid 189 does not prevent processing to a detergent partitioning form, although processing is slowed by ~60%. Therefore, only if microsomes are present in the lysate is amino acid 189 removed from the processed K-ras protein, indicating that the -AAX proteolytic activity is, like the methyltransferase, associated with the microsomal membranes. The complete loss of all the label at amino acid 189 suggests that the protease present in the added microsomal membranes is sufficient to fully process all of the prenylated protein. A repeat of this experiment in the presence of 3 mM MTA showed that the methyltransferase inhibitor did not block the proteolytic removal of amino acid 189W (data not shown).

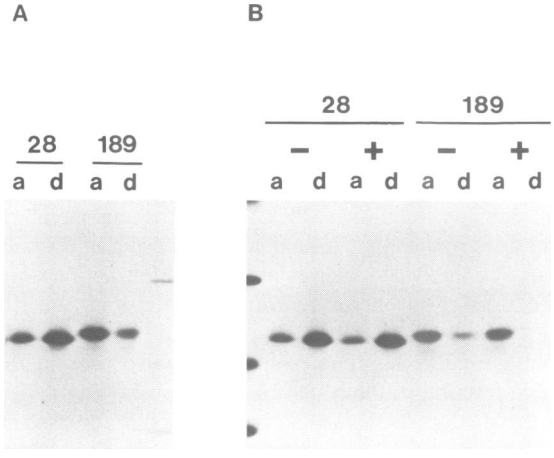

Fig. 4. The -AAX protease is membrane associated. **A**. Translations of 28W and 189W substituted p21$^{K\text{-}ras(B)}$ proteins were labelled with [^{35}S]methionine. The lysates were supplemented with MVA but the translations were performed without microsomes. Aliquots of the reactions were partitioned in Triton X-114 and the aqueous (a) and detergent (d) phases immunoprecipitated. The presence of a tryptophan residue at amino acid 189 reduces the detergent partitioning fraction from 70% (wild-type control) to 30%. **B**. Translations of 28W and 189W substituted p21$^{K\text{-}ras(B)}$ proteins, labelled with [^{3}H]tryptophan, were carried out in the absence (−) or presence (+) of microsomal membranes in lysates supplemented with MVA. Aliquots of the reactions were partitioned in Triton X-114 and the aqueous (a) and detergent (d) phases immunoprecipitated.

Prenylation alone is insufficient for efficient membrane binding

Given that *in vitro* translation, depending on the presence of microsomes, could be used to produce both fully and partially processed forms of p21$^{K\text{-}ras(B)}$ it was of interest to assess the relative importance to membrane binding of the various CAAX modifications. The proportion of processed p21$^{K\text{-}ras(B)}$ present in the lysate was first determined by Triton X-114 partitioning, immunoprecipitation and SDS−PAGE. This estimation is necessary since only processed p21^{ras} binds to membranes *in vivo* (Hancock *et al.*, 1989). Varying amounts of reticulocyte lysate were incubated with P100 membrane fractions prepared from COS cells. The P100 fractions were then reisolated by

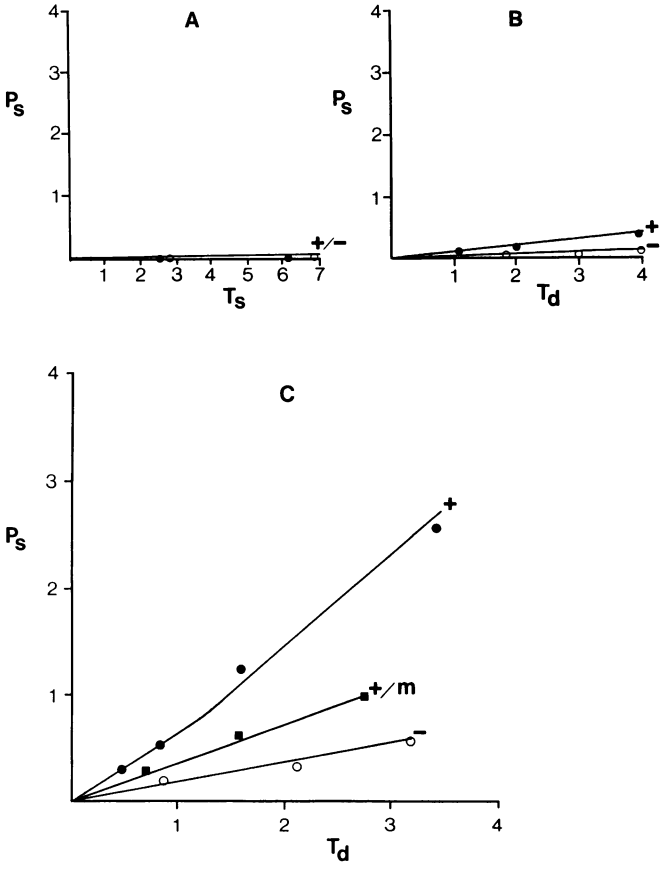

Fig. 5. Membrane binding of *in vitro* processed K-ras(B) proteins. Translations of p21$^{K\text{-}ras(B)}$ proteins were performed in the presence (+) and absence (−) of pancreatic microsomes. Labelling was with [^{35}S]methionine in MVA supplemented lysates. For wild-type p21$^{K\text{-}ras(B)}$ translations were also performed with microsomes in the presence of 3 mM MTA (+/m). Varying amounts of the lysates were incubated with P100 fractions prepared from COS cells and the amount of p21$^{K\text{-}ras(B)}$ bound to the acceptor membranes determined as described (Materials and methods). The amount of p21$^{K\text{-}ras(B)}$ binding specifically to the P100 acceptor membranes (P_S) is plotted as c.p.m. × 10^{-5} along the *y* axis. The amount of p21$^{K\text{-}ras(B)}$ incubated with the membranes is given as T_D (=detergent partitioning soluble ras protein) or, for the K186 protein, T_S (=total soluble ras protein). T_D and T_S are plotted on the *x* axis as c.p.m. × 10^{-5}. The results of an individual experiment are shown on the graphs, but each of the plotted lines summarizes data pooled from 4−8 independent experiments. **A** p21$^{K\text{-}ras(B)}$ Ser 186, **B** p21$^{K\text{-}ras(B)}$ [(Lys175−180) → Gln] = K6Q, **C** p21$^{K\text{-}ras(B)}$.

Table I. Summary of C-terminal processing in a rabbit reticulocyte lysate

Construct	Micro	MTA	C-terminus	F_D	Membrane binding
K186S	–	–	$--S\ V\ I\ M(^-)$	0%	0%
K wt	–	–	$--C\ V\ I\ M(^-)$ | C_{15}	70%	20%
K wt	+	+	$--C(^-)$ | C_{15}	70%	40%
K wt	+	–	$--C^{-Me}$ | C_{15}	70%	60–80%

The table gives C-terminal structures of K-ras proteins translated in reticulocyte lysates, supplemented with MVA, depending on the presence of microsomes and/or MTA. F_D is the proportion of the protein partitioning into the detergent phase of Triton X-114 under the specified reaction conditions and the final column gives the extent of membrane association of the processed protein. K wt = wild-type $p21^{K\text{-}ras(B)}$, K186S = $p21^{K\text{-}ras(B)}$ Cys186 → Ser, (–) = free C-terminal α-carboxyl group, $^{-Me}$ = methylesterified α-carboxyl group, C_{15} = farnesyl.

centrifugation at 100 000 *g* and the amount of $p21^{K\text{-}ras(B)}$ bound determined (legend to Figure 5). Following translation, Triton X-100 was added to the lysates to a final concentration of 0.05% in order to maximize the proportion of soluble protein. In subsequent incubations of lysate with acceptor membranes the final concentration of Triton was adjusted to 0.02%. In similar experiments with $pp60^{src}$, Resh (1989) has shown that 0.02% Triton has no detrimental effect on the acceptor membranes. Control centrifugations performed in the absence of added P100 fraction showed that ~5% of $p21^{K\text{-}ras(B)}$ protein, translated in the absence of microsomes, was insoluble under these reaction conditions. This fraction increased to ~15% in translations performed in the presence of microsomes. There was, however, no evidence to suggest that $p21^{K\text{-}ras(B)}$ was binding to the microsomes since the pellet contained aqueous and detergent partitioning forms of $p21^{K\text{-}ras(B)}$ in similar proportions to those found in the soluble fraction. Thus the pellet was not significantly enriched for processed $p21^{K\text{-}ras(B)}$.

Figure 5 shows the membrane binding of $p21^{K\text{-}ras(B)}$ proteins that have been processed in the presence and absence of microsomes. None of the K186 control protein bound to the P100 membranes in the conditions of this assay (Figure 5A) and <10% of processed K6Q protein bound to the acceptor membranes whether or not the protein was proteolysed and methylated (Figure 5B). This result is consistent with the observed distribution of the K6Q mutant protein *in vivo* (Hancock *et al.*, 1990).

Farnesylated, non-proteolysed, non-methylated wild-type $p21^{K\text{-}ras(B)}$ did bind to the P100 membranes but only inefficiently; Figure 5C shows that <20% of the farnesylated protein bound to the acceptor membranes over a wide range of $p21^{K\text{-}ras(B)}$ concentrations. However, $p21^{K\text{-}ras(B)}$ protein translated in the presence of microsomal membranes bound much more extensively to the P100 membranes. Figure 5C shows that 60–80% of the fully processed $p21^{K\text{-}ras(B)}$ present in the lysate bound to the acceptor membranes over the range of $p21^{K\text{-}ras(B)}$ concentrations investigated. This figure compares favourably with the proportion of $p21^{K\text{-}ras(B)}$ found associated with the P100 fraction *in vivo* (Hancock *et al.*, 1990). Approximately 40% of the $p21^{K\text{-}ras(B)}$ processed in the presence of microsomes and MTA bound to the acceptor membranes (Figure 5C). Thus farnesylated, -AAX proteolysed, predominantly non-methylated $p21^{K\text{-}ras(B)}$ binds less extensively to membranes than the fully methylated protein.

Finally, to exclude the possibility that the endogenous SAM concentration was limiting for full methylation of the protein, $p21^{K\text{-}ras(B)}$ was translated with added microsomes in lysates supplemented with both MVA and cold SAM. Adding SAM to a final concentration of 50 μM produced no increment in the P100 binding of $p21^{K\text{-}ras(B)}$ protein translated and processed in such modified lysates. However, SAM concentrations of 100 μM or above significantly inhibited translation.

It is also of interest to note that ~32 μg of microsomal membranes are added to 50 μl of reticulocyte lysate in this protocol. Therefore binding of $p21^{K\text{-}ras(B)}$ to 4 μg of acceptor plasma membrane is carried out in the presence of up to an 8-fold excess of intracellular membranes; a striking demonstration of the specificity of $p21^{K\text{-}ras(B)}$ binding to plasma membrane.

Discussion

The plasma membrane targeting of $p21^{ras}$ requires the C-terminal CAAX motif and a second signal of either palmitoylation or a polybasic motif. The requirement for the second signal in directing plasma membrane targeting has been addressed *in vivo* using mutant ras proteins (Hancock *et al.*, 1990). Dissecting the individual roles of farnesylation, AAX proteolysis and methylesterification in plasma membrane targeting is difficult *in vivo* since these modifications appear to be very closely coupled and there are no specific inhibitors for proteolysis and methylation. Furthermore mutations introduced into the CAAX motif block all processing by preventing farnesylation, which is the first post-translational modification of $p21^{ras}$ (Hancock *et al.*, 1989).

We have shown here that farnesylation, proteolysis and methylation of $p21^{K\text{-}ras(B)}$ are possible in a reticulocyte lysate supplemented with MVA and microsomal membranes. Farnesylpyrophosphate transferase is present in the soluble fraction of the lysate, as it is in the soluble fraction of other cell extracts (Vorburger *et al.*, 1990; Reiss *et al.*, 1990). By implication from the results presented here, the synthetic enzymes of the isoprene pathway are also present in the soluble fraction of the lysate. It has been shown previously that the methyltransferase activity is membrane associated (Stephenson and Clarke, 1990); our results show that the -AAX protease is also found on intracellular membranes. This is consistent with recent work on the processing of C-terminal peptides of $p21^{ras}$ in soluble *in vitro* systems. The peptides were found to be modified by the addition, predominantly of C_{15} farnesol, but were not proteolysed or methylesterified (Schafer *et al.*, 1990).

It is interesting to note that the processing of $p21^{K\text{-}ras(B)}$ in this *in vitro* system is very rapid and clearly exhausts the available supply of MVA unless extra is provided. We have noted previously that the processing of $p21^{K\text{-}ras(B)}$ *in vivo* is much more rapid than that of $p21^{H\text{-}ras}$ and $p21^{N\text{-}ras}$ (Hancock *et al.*, 1989). We have also observed that the

processing of p21$^{H\text{-}ras}$ is much slower than that of p21$^{K\text{-}ras(B)}$ in the reticulocyte lysate to the extent that the endogenous supply of MVA is not limiting over the course of the incubation (data not shown). The recent observation that the p21$^{K\text{-}ras(B)}$ CAAX peptide is a much better substrate for farnesyltransferase than the p21$^{H\text{-}ras}$ CAAX peptide (Reiss *et al.*, 1990) rationalizes these data. Similarly the presence of a tryptophan residue at amino acid 189 slows processing presumably by making the modified CAAX sequence a poorer substrate for the farnesyltransferase.

The data presented here, summarized in Table I, show that farnesylation alone increases the hydrophobicity of p21$^{K\text{-}ras(B)}$ sufficiently to cause partitioning into the detergent phase of Triton X-114. However, farnesylated, non-proteolysed, non-methylated p21$^{K\text{-}ras(B)}$ associates inefficiently with cell membranes. Removal of the -AAX amino acids produces a 2-fold increment in the extent of membrane binding *in vitro*, and subsequent methylation results in a further 2-fold increment in membrane binding (Table I). These experiments also show that, as in the intact cell, the combination of a processed CAAX motif and a polybasic domain is essential for the *in vitro* membrane binding of p21$^{K\text{-}ras(B)}$.

The interesting question arising from these data is why 'AAXing' and methylation should significantly increase the extent of membrane binding of farnesylated p21$^{K\text{-}ras(B)}$. Removal of the last three amino acids may be required because they sterically hinder insertion of the farnesyl group into the lipid bilayer or a receptor protein. The requirement for methylation is presumably to neutralize the negative charge on the ionized carboxyl group which could cause repulsion from the negatively charged head groups of membrane phospholipids. If p21$^{K\text{-}ras(B)}$ does bind to a receptor protein in the plasma membrane, an analogy with another CAAX protein, the yeast mating factor-a, is apparent. A chemically demethylated form of the mature factor has been shown to be biologically inactive (Anderegg *et al.*, 1989), which may reflect an inability of the demethylated factor to bind efficiently to its receptor on the target cell.

Materials and methods

Plasmids and focus assays

All mutations in K-*ras*(B) cDNA were constructed using oligonucleotide directed mutagenesis and the cDNA fully sequenced before subcloning into the expression vector pGEM-9Z(f-) (Promega). RNA was translated *in vitro* using T7 DNA dependent RNA polymerase and linearized plasmid as template.

Translation reactions

Nuclease treated reticulocyte lysate was obtained from Promega, UK, and translations performed according to the manufacturer's instructions. Typically reactions were performed at 30°C for 90 min using 2 μg uncapped RNA in a reaction volume of 50 μl. Mevalonic acid lactone (Sigma) was converted to MVA by alkalkine hydrolysis as described (Kita *et al.*, 1980), stored as an aqueous 100 mM stock solution at −20°C and 2.5−5 μl added to the translation reactions where indicated. Canine pancreatic microsomal membranes (Promega) were stored at −70°C and thawed on ice immediately before use. 7.2 EQ of membranes (3.6 μl) were used in a 50 μl translation reaction. Following translation, 2.5 μl of 1% Triton X-100 in NT buffer (50 mM Tris−Cl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$) was added to adjust the final Triton concentration to 0.05%.

Methionine free amino acid mix (Promega) was used for the labelling of translations with [^{35}S]methionine (Amersham, SJ204; at 1.2 mCi/ml^{-1} final concentration) in 50 μl reactions. Appropriate 1 mM L-amino acid (Sigma) mixes were prepared for labelling translation reactions with MVA and *S*-adenosyl-methionine (all 20 amino acids) or tryptophan (19 amino acids minus tryptophan). These reactions were labelled with 50 μCi R-[2-^{14}C]mevalonic acid lactone (Amersham, CFA660) after removing the benzene solvent under a stream of nitrogen at 37°C, or 100 μCi SAM (ICN, 24051H) or 25 μCi [5-^{3}H]tryptophan (NEN, NET782) after removing the ethanol/H_2SO_4 and water/ethanol solvents respectively under a vacuum at −60°C. The dried label was taken up directly in reticulocyte lysate in each case and the translations performed in a final volume of 25 μl. MTA (Sigma) was kept as a 0.5 M stock in DMSO at −20°C and thawed immediately before use.

Triton X-114 partitioning and immunoprecipitations

2 μl of a [^{35}S]methionine-labelled reaction, or 25 μl of a [^{3}H]tryptophan or [^{14}C]MVA labelled translation reaction were taken up in 1 ml of ice cold 1% Triton X-114, warmed to 37°C for 2 min and the aqueous and detergent phases separated by a 2 min microfuge spin (Gutierrez *et al.*, 1989; Hancock *et al.*, 1989). Immunoprecipitations with Y13-259 and resolution of the proteins by SDS−PAGE were performed as described (Gutierrez *et al.*, 1989; Hancock *et al.*, 1988). Gels were soaked in Enlightening (NEN) before being autoradiographed at −70°C. ^{35}S-Labelled gels were autoradiographed for 4−12 h and ^{3}H- and ^{14}C-labelled gels were autoradiographed for 7−28 days.

To measure the radioactivity in immunoprecipitated p21^{ras}, autoradiograms were used as guides to cut out gel slices containing the ras protein. These were re-hydrated and digested with proteinase K (100 μg/ml) in Tris buffered saline at 37°C for 48 h, the gel slice and proteinase K solution were taken up in scintillation fluid and counted. The proportion of processed p21^{ras} in an aliquot of lysate (= F_D) is then calculated as (c.p.m. in p21^{ras} partitioning into detergent)/(total c.p.m. in p21^{ras}).

Membrane binding assays

P100 fractions were prepared from COS cells as described (Cales *et al.*, 1988), adjusted to a protein concentration of 0.5 μg/μl and stored in NT buffer in aliquots at −70°C until required. Reticulocyte lysate (5−60 μl) was incubated with 8 μl (4 μg) P100 fraction at 20°C for 30 min. The volume of the reaction was 50 μl (for 20 μl lysate or less) or the minimum volume, required for a final Triton X-100 concentration of 0.02%. In reactions using >20 μl lysate, Triton X-100 concentrations were adjusted so that the final concentration was always 0.02%. After the 30 min incubation the P100 fraction was reisolated by centrifugation at 100 000 *g* at 4°C for 30 min. The S100 fraction was removed and 5× concentrated SDS sample buffer added. The P100 pellet was taken up into 1 × SDS sample buffer. Both fractions were boiled and 20−50% of the sample resolved by SDS−PAGE. The amount of radioactivity in p21^{ras} in each sample was then determined as described above. The amount of insoluble p21^{ras} present was determined by carrying out a control incubation in the absence of added P100 fraction. The c.p.m. present in insoluble p21^{ras} were then subtracted from the c.p.m. isolated in the presence of P100 (in proportion to the amount of p21^{ras} present in the reaction) to determine the amount of p21^{ras} binding specifically to the acceptor membranes (P_S).

The processed p21^{ras} available in an incubation to bind to the acceptor membranes (T_D = total detergent partitioning c.p.m.) was calculated as (total c.p.m. in S100 + total c.p.m. in P100 − insoluble c.p.m.) × F_D.

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Note added in proof

We have now demonstrated by methyliodide cleavage (Casey *et al.*, 1989) and HPLC analysis that the isoprenoid covalently bound to $p21^{K\text{-}ras(B)}$ following *in vitro* translation is C_{15} farnesyl