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A CAAX or a CAAL motif and a second signal are sufficient for plasma membrane targeting of ras proteins

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Mutational analysis of $p21^{ras}$ has shown that plasma membrane targeting requires the combination of a CAAX motif with a polybasic domain of six lysine residues or a nearby palmitoylation site. However, it is not known from these studies whether these signals alone target $p21^{ras}$ to the plasma membrane. We now show that these C-terminal sequences are sufficient to target a heterologous cytosolic protein to the plasma membrane. Interestingly, the key feature of the $p21^{K\text{-}ras(B)}$ polybasic domain appears to be a positive charge, since a poly-arginine domain can function as a plasma membrane targeting motif in conjunction with the CAAX box and $p21^{K\text{-}ras(B)}$ with the polylysine domain replaced by arginines is biologically active. Since some ras-related proteins are modified by geranylgeranyl rather than farnesyl we have investigated whether modification of $p21^{ras}$ with geranylgeranyl affects its subcellular localization. Geranylgeranyl can substitute for farnesyl in combining with a polybasic domain to target $p21^{K\text{-}ras(B)}$ to the plasma membrane, but such geranylgeranylated proteins are more tightly bound to the membrane. This increased avidity of binding is presumably due to the extra length of the geranylgeranyl alkyl chain.

Key words: CAAX/plasma membrane/prenylation/*ras*/targeting

Introduction

The $p21^{ras}$ proteins are localized to the inner surface of the plasma membrane (Willingham *et al.*, 1980). Mutational analysis has shown that one element of plasma membrane localization is an intact CAAX motif at the C terminus of the protein (Willumsen *et al.*, 1984; Hancock *et al.*, 1989). A CAAX motif (C = cysteine, A = aliphatic, X = any amino acid) is found at the C terminus of all ras proteins and many other cellular proteins. The motif undergoes a triplet of closely coupled post-translational modifications. Firstly, a prenoid derivative is linked as a thioether to the cysteine residue (Hancock *et al.*, 1989; Casey *et al.*, 1989); second, the -AAX amino acids are removed by proteolysis (Gutierrez *et al.*, 1989) and third, the α-carboxyl group of the now C-terminal cysteine residue is methyl-esterified (Clarke *et al.*, 1988; Gutierrez *et al.*, 1989). We have shown recently that all three of these post-translational processing events at the CAAX motif are required for efficient membrane binding of $p21^{K\text{-}ras(B)}$ (Hancock *et al.*, 1991).

The $p21^{ras}$ proteins (Casey *et al.*, 1989), nuclear lamin B (Farnsworth *et al.*, 1989) and the γ-subunit of transducin (Fukada *et al.*, 1990; Lai *et al.*, 1990) are all prenylated with C_{15} farnesyl. Certain other CAAX containing proteins, including the ras-related proteins K*rev*1/*rap*1A (Kawata *et al.*, 1990; Buss *et al.*, 1991) and G25K (Maltese and Sheridan, 1990), and the γ-subunits of brain G-proteins (Yamane *et al.*, 1990; Mumby *et al.*, 1990) have been shown to be geranylgeranylated. The CAAX motifs of these C_{20} modified proteins all terminate with a leucine residue indicating that the X amino acid determines whether a CAAX motif is a substrate for a farnesyl or geranylgeranyl transferase (Seabra *et al.*, 1991; Finegold *et al.*, 1991). It is not known why some proteins are farnesylated and others are geranylgeranylated nor whether the alkyl chain length affects subcellular localization or avidity of membrane association.

The modifications of the CAAX motif provide only one part of the signal for the subcellular localization of proteins to cellular membranes. The CAAX motif of $p21^{ras}$ combines with a second signal contained within the C-terminal hypervariable domain of ras proteins to target plasma membrane localization. This second signal comprises either a cysteine palmitoylation site in the case of $p21^{H\text{-}ras}$, $p21^{N\text{-}ras}$ and $p21^{K\text{-}ras(A)}$, or a polybasic domain comprising six consecutive lysine residues (amino acids 175-180) in the case of $p21^{K\text{-}ras(B)}$ (Hancock *et al.*, 1990). Similarly, the nuclear lamins A and B require both a CAAX motif and a nuclear localization signal for correct targeting to the nuclear membrane (Holtz *et al.*, 1989).

While mutational analysis of $p21^{ras}$ demonstrates that both the CAAX motif and either palmitoylation or a polybasic domain are necessary for plasma membrane localization of $p21^{ras}$ it is not clear whether the combination of these signals is sufficient for plasma membrane localization. It is possible that there are other domains which are also required. To address this question we have investigated whether a heterologous cytosolic protein, protein A, can be targeted to the inner surface of the plasma membrane by a CAAX motif in combination with a polybasic domain or a palmitoylation signal. We have also studied whether other positively charged amino acid sequences can substitute for the wild type polylysine tract of $p21^{K\text{-}ras(B)}$ as subcellular targeting motifs and determined whether CAAX motifs which direct geranylgeranylation can target $p21^{ras}$ and protein A to the inner surface of the plasma membrane.

Results

Plasma membrane targeting of protein A

In the light of our previous studies using a mutational analysis (Hancock *et al.*, 1989, 1990) it was of interest to determine whether the polybasic domain of $p21^{K\text{-}ras(B)}$ together with a CAAX motif could function as a plasma membrane targeting sequence for an heterologous protein. Using polymerase

chain reaction (PCR) the coding sequence for the C-terminal 17 amino acids of $p21^{K\text{-}ras(B)}$ was amplified from a K-*ras(B)* cDNA and cloned onto the C terminus of protein A using a restriction site introduced into the K-*ras(B)* coding sequence during amplification. Figure 1 shows that the chimeric protein expressed in COS cells localized entirely to the P100 fraction on subcellular fractionation, whereas protein A without $p21^{K\text{-}ras(B)}$ C-terminal sequences localized predominantly (80%) to the S100 fraction (Figure 1). The addition of the 17 C-terminal amino acids of K6Q, a mutant $p21^{K\text{-}ras(B)}$ protein with the polylysine domain replaced with six glutamine residues (Hancock *et al.*, 1990), did not shift protein A into the P100 fraction (Figure 1).

The subcellular localization of the protein A chimeras was further analysed by immunofluorescent studies in MDCK

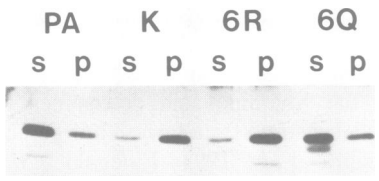

Fig. 1. COS cells expressing protein A (PA), or protein A chimeras with the C-terminal 17 amino acids from K-ras(B) (K), K6Q (6Q) and K6R (6R) were fractionated into P100 (p) and S100 (s) fractions. The polylysine domain of wild type K-ras(B) is replaced with polyglutamine in the K6Q mutant protein and with polyarginine in the K6R mutant K-ras(B) protein. Equal proportions of the S100 and P100 fractions were resolved by SDS–PAGE and Western blotted using an anti-protein A primary antibody.

cells transiently expressing the proteins following microinjection of plasmid DNA. Figure 2 shows that protein A with wild type $p21^{K\text{-}ras(B)}$ C-terminal sequences localized to the plasma membrane and protein A with K6Q C-terminal sequences localized to the cytosol. The only observable effect of a CAAX motif in the absence of a polybasic domain was to exclude protein A from the nucleus (Figure 2). Together the data from the cell fractionation experiments and the immunofluorescence localization shows that a polylysine domain can combine with a CAAX motif to target a heterologous protein to the plasma membrane but a farnesylated CAAX motif in isolation does not lead to plasma membrane (or any other membrane) association. Similarly, when the C-terminal 10 amino acids of $p21^{H\text{-}ras}$ comprising two cysteine palmitoylation sites plus a CAAX motif were cloned into the C terminus of protein A, the chimeric protein localized to the plasma membrane (Figure 3). However, if both of the cysteine residues required for palmitoylation are replaced with serines then the protein is cytosolic just like the protein A–K6Q chimera (Figure 2).

We next analysed the ability of polybasic domains with fewer than six Lys → Gln substitutions to function as plasma membrane targeting motifs. Figure 4 shows that as the number of lysine residues within the polybasic domain was progressively reduced, the protein A chimeras were increasingly localized to the cytosol. No plasma membrane localization occurs when there are fewer than two lysines in the polybasic domain. Thus the polybasic domain functions for a heterologous protein exactly as for $p21^{K\text{-}ras(B)}$ (Hancock *et al.*, 1990).

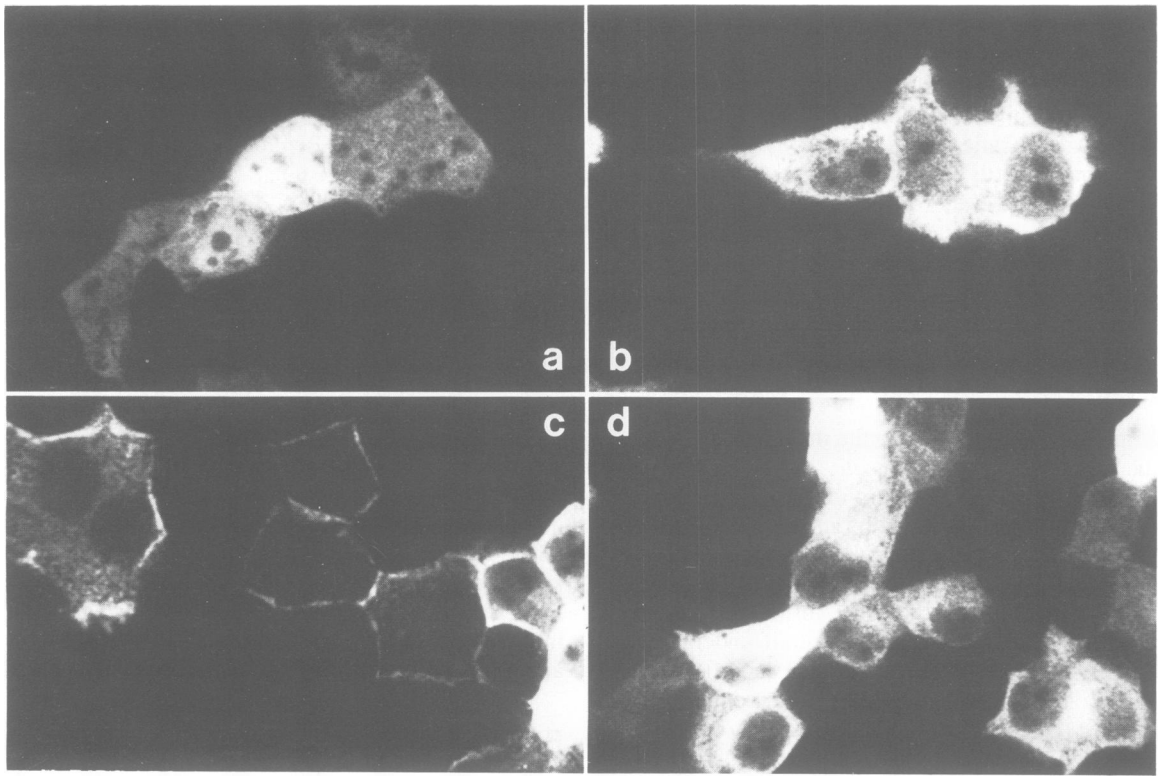

Fig. 2. Immunofluorescent analysis of MDCK cells transiently expressing protein A or protein A chimeric proteins following microinjection of plasmid DNA. (**a**) Protein A: cytoplasmic and nuclear staining. (**b**) Protein A with a CVLS C-terminal motif alone (from $p21^{H\text{-}ras}$): cytoplasmic staining only. (**c**) Protein A with 17 C-terminal amino acids from wild type $p21^{K\text{-}ras(B)}$: plasma membrane staining. (**d**) Protein A with 17 C-terminal amino acids from the K-ras(B) mutant, K6Q, which has the polylysine domain replaced with polyglutamine: cytoplasmic staining only.

Polyarginine can substitute for polylysine as a plasma membrane targeting motif

It is possible that the polylysine domain could function as part of a plasma membrane targeting signal in two ways. First, since the domain is positively charged at physiological pH, an electrostatic interaction with negatively charged phospholipid head groups may be important. Second, there may be a specific docking protein for $p21^{K\text{-}ras(B)}$ in the plasma membrane with which the polylysine domain interacts. If the second model is true it is probable that substituting other positively charged amino acids for the lysine residues would comprise the function of the domain. To address these possibilities we constructed a mutant $p21^{K\text{-}ras(B)}$ protein, K6R, which has six Lys → Arg substitutions at amino acids 175–180.

The biological activity of oncogenic mutant K6R (Gly12 → Val) was tested in focus assays on NIH3T3 cells, and found to be similar to $p21^{K\text{-}ras(B)}$ (Gly12 → Val) (Table I). Immunofluorescence studies on NIH cell lines transformed by the K6R mutant showed that the K6R protein localized to the plasma membrane (data not shown) and subcellular fractionation of COS cells expressing the protein showed >90% localization to the P100 fraction. The 17 C-terminal amino acids of K6R were then cloned onto the C terminus of protein A and the chimeric protein expressed in COS and MDCK cells. Subcellular fractionation demonstrated that the chimeric protein was >90% P100 associated (Figure 1) and the immunofluorescence studies presented in Figure 4 show that the protein A–K6R chimera localized to the plasma membrane of MDCK cells.

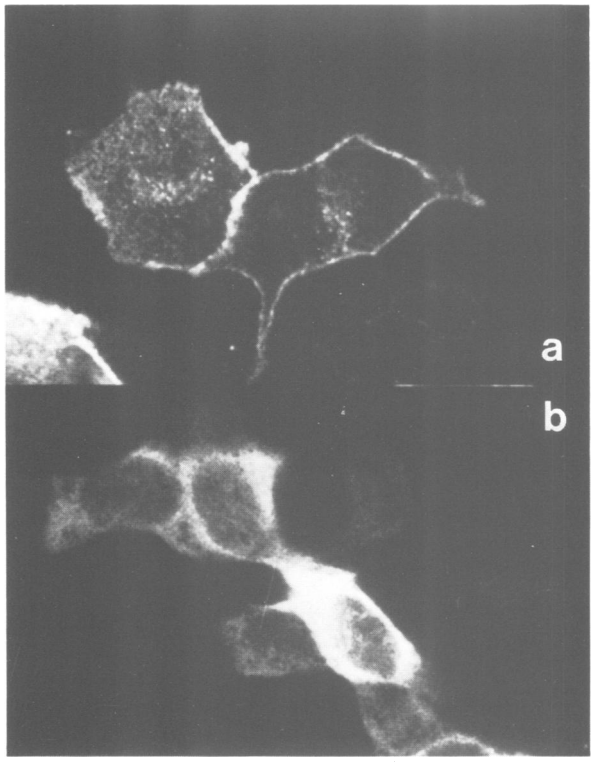

Fig. 3. Immunofluorescent analysis of MDCK cells transiently expressing protein A or protein A chimeric proteins following microinjection of plasmid DNA. (**a**) Protein A with the C-terminal 10 amino acids from $p21^{H\text{-}ras}$: plasma membrane staining. (**b**) Protein A with the C-terminal 10 amino acids from $p21^{H\text{-}ras}$ Cys181, Cys184 → Ser, this protein A chimera is therefore not palmitoylated: cytoplasmic staining only (compare with Figure 2d).

Analysis of the subcellular localization of geranylgeranylated $p21^{K\text{-}ras(B)}$

So far the only ras-related protein with a C_{20} geranylgeranyl modification that has had its intracellular localization determined is rap1. Both rap1A and rap1B have C-terminal polybasic domains like $p21^{K\text{-}ras(B)}$ and Beranger *et al.* (1991) have shown that antibodies against rap1 stain the Golgi. It was therefore of interest to determine whether a C_{20} modification of $p21^{K\text{-}ras(B)}$ would alter its subcellular localization. Two K-*ras(B)* constructs were made which changed the wild type CVIM sequence to CAIL, the CAAX motif of a brain G-protein γ-subunit and CCIL, the CAAX motif of *ral*. The same CAAX box mutations were also made in the K6Q K-*ras(B)* mutant.

To confirm that the $p21^{K\text{-}ras(B)}$ CAIL and CCIL proteins were modified by geranylgeranylation, they were translated *in vitro* in a rabbit reticulocyte lysate labelled with [^{3}H]mevalonic acid (Hancock *et al.*, 1991). Following SDS–PAGE and fluorographic detection the ras proteins were excised from the polyacrylamide gel, digested out of the gel slices and subjected to methyliodide cleavage (Casey *et al.*, 1989). The products of the cleavage were analysed by HPLC. For both proteins the counts retained on the column co-eluted with the C_{20} geranylgeraniol standard and no label was detected in the position of farensol. In addition, both the CAIL and CCIL proteins incorporated label from *S*-adenosyl-[^{3}H]methyl-methionine when translated *in vitro* in the presence of microsomal membranes (data not shown). Thus the CAIL and CCIL mutant $p21^{K\text{-}ras(B)}$ proteins are geranylgeranylated and methylesterified. A recent study of the post-translational processing of full length *ral* (Kinsella *et al.*, 1991) also found that the CCIL motif was geranylgeranylated. These data are therefore consistent with the hypothesis that CAA(X = L) motifs direct geranylgeranylation rather than farnesylation. Such motifs are also methylated and by implication they must be -AA(X = L) proteolysed.

We next investigated whether the presence of a C_{20} alkyl chain affected the subcellular distribution of the $p21^{K\text{-}ras(B)}$ protein. Fractionation of COS cells expressing the CAIL and CCIL proteins showed that they were localized to the P100 fraction (Figure 5) and immunofluorescence studies of NIH3T3 cells expressing the proteins showed strong plasma membrane staining (Figure 6). These results demonstrate that the presence of a C_{20} rather than a C_{15} alkyl group has little effect on the localization of K-ras(B) proteins with an intact polybasic domain. However, Figure 5 shows that a C_{20} alkyl group profoundly altered the subcellular distribution of K-ras(B) proteins in which the polybasic domain has been changed to six uncharged glutamine residues. Subcellular fractionation of COS cells expressing the geranylgeranylated K6QCAIL and K6QCCIL proteins (with the mutations Lys175–180 → Gln) revealed that they were >90% localized to the P100 fraction whereas the farnesylated K6Q protein is >90% localized to the S100 fraction (Figure 5 and Hancock *et al.*, 1990). Interestingly, examination by immunofluorescence of NIH3T3 cells expressing K6QCAIL and K6QCCIL revealed that the proteins were not localized to the plasma membrane (Figure 6). Thus while the presence

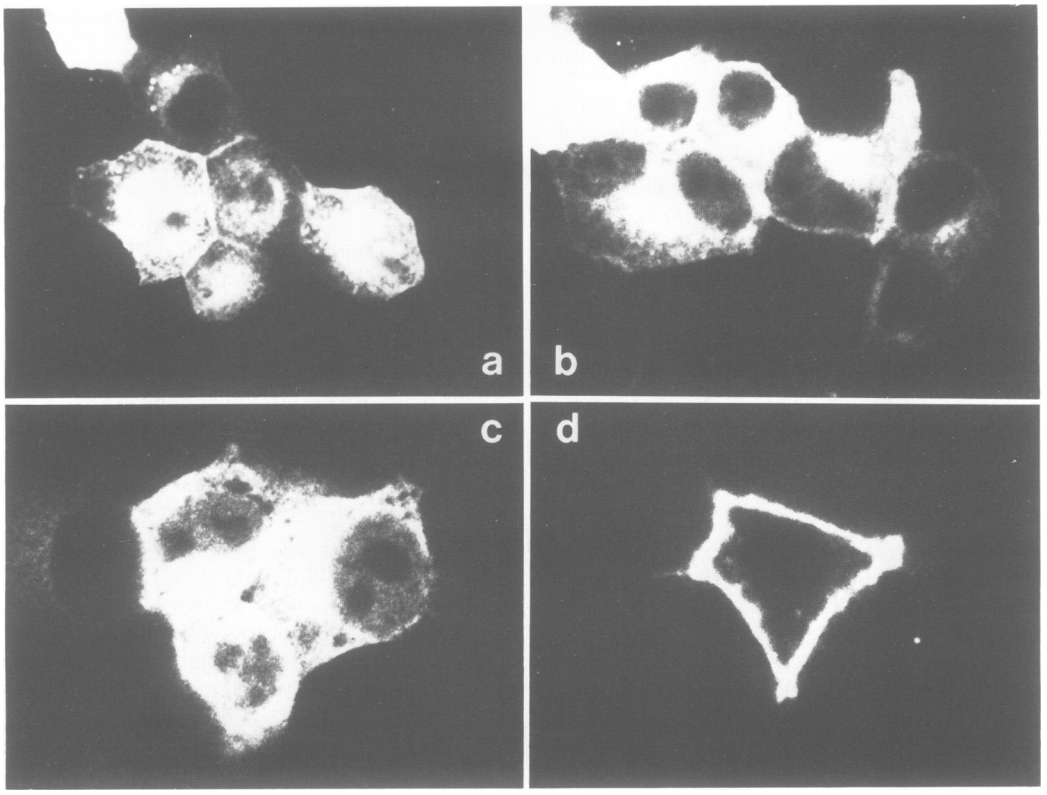

Fig. 4. Immunofluorescent analysis of MDCK cells transiently expressing protein A or protein A chimeric proteins following microinjection of plasmid DNA. (**a**) Protein A with the 17 C-terminal amino acids from a K-ras(B) protein with three glutamine substitutions in the polybasic domain: combination of plasma membrane and cytosolic staining. (**b**) Protein A with the 17 C-terminal amino acids from a K-ras(B) protein with four glutamine substitutions in the polybasic domain: predominantly cytosolic staining with weak plasma membrane staining. (**c**) Protein A with the 17 C-terminal amino acids from a K-ras(B) protein with five glutamine substitutions in the polybasic domain: cytosolic staining only. (**d**) Protein A with the 17 C-terminal amino acids from a K-ras(B) protein with the polylysine domain replaced with polyarginine: plasma membrane staining.

of a C_{20} chain leads to the association of the K6QCCIL and K6QCAIL proteins with P100 membranes, it does not restore plasma membrane association. This is in agreement with our previous observation that both a polybasic domain and a CAAX motif are required for plasma membrane localization (Hancock *et al.*, 1990). The presence of a farnesyl or geranylgeranyl chain appeared to have little effect on the biological activity of oncogenic K-ras(B) proteins, as measured in NIH3T3 transformation assays (Table I), either in the context of an intact polybasic domain or in K-ras(B) proteins with the K6Q substitutions. This result, therefore, demonstrates that for transforming activity prenylation with a C_{15} or a C_{20} alkyl chain is effective.

The C_{20} modified K-ras(B) proteins are avidly associated with the membrane pellet. Table II shows that a 1 M salt wash removes 78% of farnesylated $p21^{K\text{-}ras(B)}$ from the P100 fraction whereas $\leq 15\%$ of geranylgeranylated $p21^{K\text{-}ras(B)}$ is removed under the same conditions. The tighter membrane association of the geranylgeranylated proteins is independent of the presence of a polybasic domain. However, 0.5% Triton X-100 solubilizes >90% of all the geranylgeranylated and farnesylated K-ras(B) proteins from the P100 fraction (Table II), thus suggesting that the K6QCAIL and K6QCCIL proteins are associated with an intracellular membrane rather than a high molecular weight cytoplasmic protein complex. We are currently investigating to which intracellular membrane compartment these geranylgeranylated, polybasic mutant proteins are being targeted.

Finally, Figure 7 shows that the subcellular distribution of protein A chimeras with the 17 C-terminal amino acids from the CCIL and K6QCCIL K-ras(B) constructs is exactly the same as the parent K-ras(B) proteins. Moreover, immunofluorescence studies of MDCK cells expressing these proteins show that the protein A chimeras have the same localization as the K-ras(B) CCIL and K6QCCIL proteins in NIH3T3 cells (data not shown). Thus the membrane targeting phenotypes associated with the C_{20} modified CAA(X = L) motif are fully defined by these primary amino acid sequences.

Table I. NIH3T3 focus assays

ras construct	Relative activity
K-ras	1.0
K6R	1.0
K-ras CCIL	0.88
K-ras CAIL	0.64
K6Q	0.38
K6Q CCIL	0.64
K6Q CAIL	0.2

Relative transforming efficiencies of K-*ras(B)* (Val12) cDNAs with altered C termini. 20 ng of each EXV plasmid was transfected with 20 μg normal human DNA as carrier onto 1.3×10^5 NIH3T3 cells. Foci were scored 14–16 days following transfection. K-ras(B) (Val12) with a wild type C terminus gave 1–1.9 foci/ng and results are expressed relative to this value.
CCIL = C-terminal CCIL motif substituted for wild type CVIM.
CAIL = C-terminal CAIL motif substituted for wild type CVIM.
K6Q = $p21^{K\text{-}ras(B)}$ with the mutations Lys175–180 → Gln.

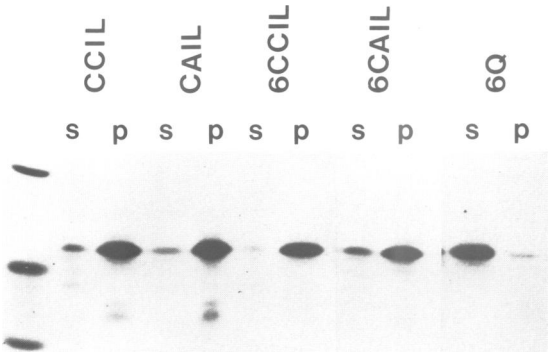

Fig. 5. COS cells expressing K-ras(B) proteins with metabolically labelled with [^{35}S]methionine for 18 h and fractionated into P100 (p) and S100 (s) fractions. Equal proportions of each fraction were immunoprecipitated, resolved by SDS−PAGE and autoradiographed. CCIL = p21$^{K\text{-}ras(B)}$ with a CCIL C-terminal motif; CAIL = p21$^{K\text{-}ras(B)}$ with a CAIL C-terminal motif; 6CCIL = p21$^{K\text{-}ras(B)}$ Lys175−180 → Gln with a CCIL C-terminal motif. 6CAIL = p21$^{K\text{-}ras(B)}$ Lys175−180 → Gln with a CAIL C-terminal motif; 6Q = p21$^{K\text{-}ras(B)}$ Lys175−180 → Gln (wild type CAAX motif).

Table II. Salt and Triton wash of COS cell membranes

ras protein	Percentage P100 washout	
	1 M NaCl	0.5% Triton
K-ras	78	92
K-ras CCIL	11	90
K-ras CAIL	13	94
K6Q CCIL	12	92
K6Q CAIL	15	95

[^{35}S]Methionine-labelled P100 fractions were prepared from COS cells expressing K-ras(B) proteins. Aliquots of these fractions were washed with 1 M NaCl for 30 min at 20°C or 0.5% Triton X-100 for 10 min on ice and the membranes repelleted at 120 000 *g*. K-ras(B) protein was immunoprecipitated from the S100 and P100 fractions, resolved by SDS−PAGE and cut out of the gel following autoradiography. The amount of K-ras(B) protein remaining in the membrane pellet and the amount washed out into the S100 fraction was then quantified by scintillation counting of pronase digests of the gel slices. Results (mean of two experiments) are presented as percentage P100 washout, calculated as (c.p.m. in ras in the S100 wash fraction) × 100%/(total c.p.m. in ras). The mutant K-ras(B) proteins are described in the legend to Table I.

Discussion

We have shown previously that a C-terminal polybasic domain, comprising six lysine residues (amino acids 175−180) of p21$^{K\text{-}ras(B)}$ and the palmitoylation sites (Cys181, Cys184) of p21$^{H\text{-}ras}$, are required in addition to the CAAX motif for plasma membrane localization (Hancock *et al.*, 1990). These experiments, however, do not address whether other protein sequences might also be required for the subcellular targeting of p21^{ras}. The data presented here exclude this possibility. The C-terminal 17 amino acids from p21$^{K\text{-}ras(B)}$ are sufficient to target the heterologous protein, protein A, to the plasma membrane. Similarly the C-terminal 10 amino acids from p21$^{H\text{-}ras}$, comprising the palmitoylation sites and the CAAX motif contain all the necessary information for the plasma membrane localization of protein A. In related experiments we have shown that the same C-terminal sequences can be used to target the cytosolic GAP protein to the plasma membrane (Huang,D., Marshall,C.J. and Hancock,J.F., in preparation). A CAAX motif in isolation however is insufficient to target proteins to the plasma membrane or any other membrane.

The sequential replacement of lysine residues 175−180 with glutamine results in a progressive loss of the targeting function of the polybasic domain of p21$^{K\text{-}ras(B)}$ (Hancock *et al.*, 1990). Increasing cytosolic localization is also seen with the protein A−K-ras chimeras as the number of glutamine substitutions within the polybasic domain increases. The simplest interpretation of these data is that the polybasic sequence operates as a membrane targeting motif via the positive charge on the side chains of the constituent lysine residues. This model is supported by the observation that a polybasic domain comprising six arginine residues and hence with the same net positive charge as six lysines can fully substitute for the wild type polybasic domain.

We have also shown here that a C_{20} geranylgeranyl moiety can substitute for C_{15} farnesyl and a target p21$^{K\text{-}ras(B)}$ to the plasma membrane implying that the chain length of the alkyl group is not important if the polybasic domain is intact. It is interesting that the geranylgeranylated p21$^{K\text{-}ras(B)}$ was not targeted to the Golgi like the rap1 proteins (Beranger *et al.*, 1991) which are geranylgeranylated and have a polybasic domain upstream of their CAA(X = L) motifs. One interpretation of these results is that there are other domains within the rap1 proteins which determine Golgi localization. Such domains may override the apparent plasma membrane localization signals located at the C terminus of the rap1 proteins. Alternatively the different spacing of the polybasic domains and the CAA(X = L) motifs of the rap1 proteins compared with these motifs in the CCIL and CAIL mutant K-ras(B) proteins may be of relevance to their different subcellular localizations.

A significant difference between geranylgeranylated and farnesylated proteins relates to their relative avidity of membrane binding. We have shown here that the presence of a 20 carbon C-terminal isoprenoid chain results in an avid membrane binding which is resistant to 1 M salt extraction. In contrast, a 15 carbon isoprenoid chain together with a polybasic domain, as in p21$^{K\text{-}ras(B)}$, leads to a weaker membrane association. The presence of an additional lipid, palmitic acid, near the C terminus, results in farnesylated p21$^{H\text{-}ras}$ binding to membranes with an avidity comparable to that of a geranylgeranylated protein (Hancock *et al.*, 1990). However, the avidity of membrane binding cannot be the sole determinant of plasma membrane association since wild type K-ras(B) protein is plasma membrane associated but bound less avidly to membranes than the K6QCCIL and K6QCAIL proteins which are not associated with plasma membrane. These data also show that there are differences in the strength of membrane association between certain ras and ras-related proteins. It is possible that these differences in membrane association reflect different functional requirements of the proteins.

The data presented here also show that replacement of the farnesyl group with geranylgeranyl results in the polybasic mutant (Lys175−180 → Gln) protein being targeted to an intracellular membrane. We have yet to establish whether the K6QCCIL and K6QCAIL mutant K-ras(B) proteins are being targeted to a specific membrane compartment; however, the immunofluorescence analyses and preliminary sucrose gradient fractionations we have performed are not consistent with a Golgi localization. It is possible, however,

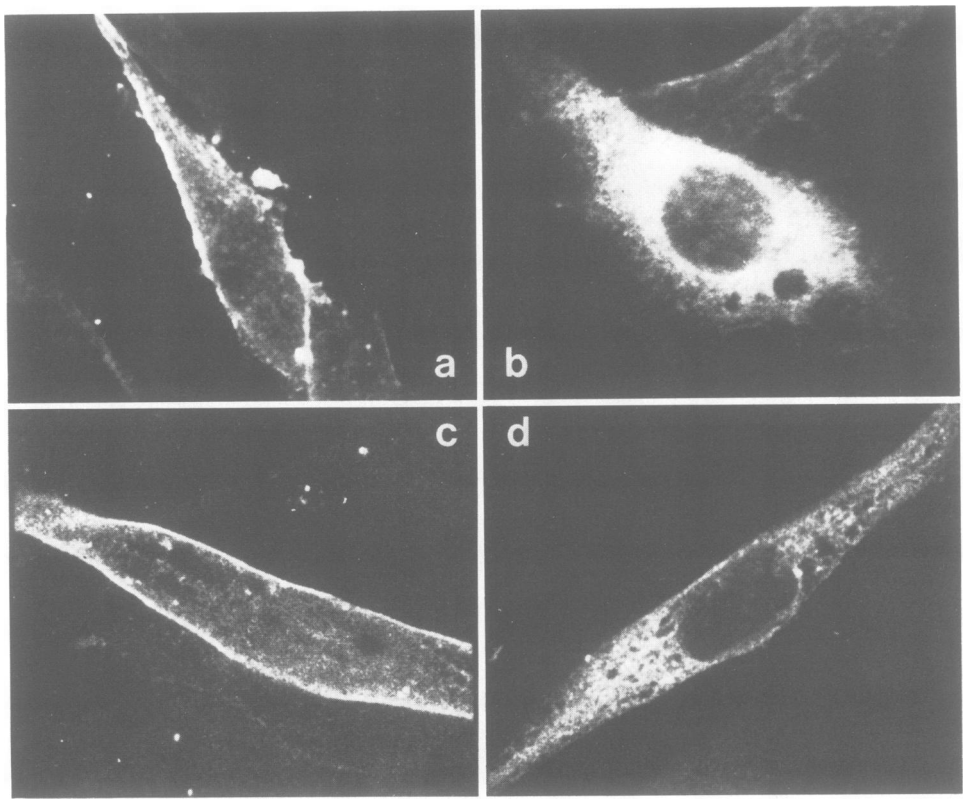

Fig. 6. NIH3T3 cell lines derived from the focus assays described in Table I were examined for immunofluorescence after incubation with the monoclonal antibody Y13-238 followed by an anti-rat FITC conjugate. This methodology allows specific staining of transfected K-ras(B) proteins [see Hancock *et al.* (1990) for a detailed description and discussion]. **(a)** $p21^{K\text{-}ras(B)}$ with a CCIL C-terminal motif: plasma membrane staining. **(b)** $p21^{K\text{-}ras(B)}$ Lys175−180 → Gln with a CCIL C-terminal motif: no plasma membrane staining, the P100 associated protein is diffusely localized through the cell. **(c)** $p21^{K\text{-}ras(B)}$ with a CAIL C-terminal motif: plasma membrane staining. **(d)** $p21^{K\text{-}ras(B)}$ Lys175−180 → Gln with a CAIL C-terminal motif: no plasma membrane staining, the P100 associated protein is diffusely localized through the cell.

given the avidity with which geranylgeranylated proteins bind to cell membranes that, in the absence of a second signal or signals directing plasma membrane (polybasic domain) or Golgi (rap1 specific domains) localization, the K6QCCIL and K6QCAIL mutant K-ras(B) proteins bind nonspecifically to all accessible intracellular membranes.

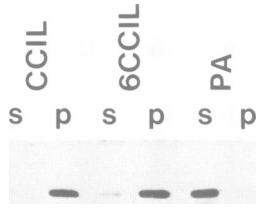

Fig. 7. COS cells expressing protein A (PA) or protein A chimeras with the 17 C-terminal amino acids from K-ras(B) mutant proteins with CCIL C-terminal motifs were fractionated into S100 (s) and P100 (p) fractions. Equal proportions of each fraction were resolved by SDS−PAGE and Western blotted with an anti-protein A primary antibody. The protein A−CCIL chimera (CCIL) has a wild type polylysine domain, whereas the protein A−6QCCIL chimera (6CCIL) has had this domain replaced with polyglutamine.

Materials and methods

Plasmids and mutagenesis

The K6R mutation was created using oligonucleotide directed mutagenesis. The tails for the protein A chimeric proteins were synthesized by PCR using the oligonucleotides 5′-ACAGAATTCAAAGATGAGCAAAGATG and 5′-AATTCTAGAGTACTAGATATGCCTTAAG to amplify a 109 bp fragment comprising the final 54 bp of the K-ras(B) coding sequence together with 37 bp of 3′ untranslated sequence plus new restriction sites (18 bp). Wild type K-ras(B) cDNA and the mutant K-ras(B) cDNAs K3Q, K4Q, K5Q, K6Q (Hancock *et al.*, 1990) and K6R were used as PCR templates. The PCR reaction was denatured for 2 min at 94°C followed by 25 cycles of 94°C for 30 s, 54°C for 45 s and 72°C for 2 min before being held at 72°C for 10 min.

The PCR product was digested with *Eco*RI and *Xba*I cloned into pGEM-9Zf(−) (Promega) for sequencing, and subsequently cloned in frame into the C-terminal polylinker of protein A in a eukaryotic expression vector (described in Hancock *et al.*, 1989).

COS cell expression

COS cells were electroporated using a method based on that described by Chu *et al.* (1987). Cells were grown to 75% confluence, harvested by trypsinization, washed twice in HeBS (20 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose) and counted. 3×10^6 cells were resuspended in 240 μl HeBS and 10 μg plasmid DNA plus 100 μg sonicated salmon sperm DNA added in a total volume of 20 μl water. The suspension was dispensed into a 0.4 cm Bio-Rad electroporation cuvette and pulsed at 250 V/125 μF (giving a time constant of ~6 ms). Cells were allowed to rest at room temperature for 10 min before seeding to 100 mm tissue culture dishes.

COS cells were harvested 72 h after transfection and fractionated as described by Cales *et al.* (1988) except that membranes were pelleted at 120 000 *g* for 30 min. Where required, cells were metabolically labelled for 18 h prior to harvesting with 100 μCi/ml Expre[^{35}S][^{35}S] labelling mix (NEN, NEG072) in methionine-free DMEM. Immunoprecipitations of fractionated COS cells, quantification of immunoprecipitated $p21^{ras}$ and salt washing of P100 fractions were performed as described previously (Hancock *et al.*, 1989, 1990).

Focus assays

NIH3T3 focus assays were performed as described by Marshall *et al.* (1981) using 20 μg normal human placental DNA as carrier and 20 ng plasmid DNA. Foci were counted 14 days after transfection.

Western blotting

The protein content of COS cell S100 fractions was determined by the Bradford reaction. 3 μg of a S100 fraction and an equal proportion of the P100 fraction were resolved by SDS−PAGE in 15% gels and the proteins transferred to nitrocellulose using a semi-dry blotting system (Pharmacia). The filter was blocked overnight in PBS-T (phosphate-buffered saline with 0.1% Tween 20) containing 7.5% (w/v) dried milk, washed briefly in PBS-T and incubated for 1 h with an anti-protein A antibody (Sigma, P3775) diluted 1:1500 in PBS-T. After washing the filter was incubated for 1 h with an anti-rabbit horseradish peroxidase conjugate (Amersham, NA9340) diluted 1:1000 in PBS-T and developed using an ECL detection kit (Amersham) according to the manufacturer's instructions. Exposure times on Hyperfilm-ECL (Amersham) ranged from 10 s to 10 min.

In vitro translations and isoprenoid analysis

R-[5-^{3}H]mevalonic acid 50 μCi (NEN, NET716) was dried under vacuum at −60°C and taken up in 50 μl nuclease-treated rabbit reticulocyte lysate (Promega) containing all 20 amino acids. Uncapped RNA (2 μg) was added and translation performed at 30°C for 90 min. The whole lysate was partitioned in Triton X-114 and the detergent partitioning fraction precipitated with 10% TCA. After incubation on ice for 1 h the precipitated proteins were collected by centrifugation, washed three times with 1 ml cold acetone, dried and taken up in Laemmli sample buffer. Following SDS−PAGE the gel was soaked in Enlightening (NEN) and autoradiographed. The labelled bands were digested with pronase from gel slices excised using the autoradiogram as a guide. 3×10^5 c.p.m. of labelled peptide were cleaved with methyl iodide using the method of Casey *et al.* (1989) and analysed by HPLC as previously described (E.Fawell, J.F.Hancock, T.Giannakouros, C.Newman, J.Armstrong and A.I.Magee, submitted).

Labelling of *in vitro* translates with *S*-adenosyl[^{3}H]methyl-methionine was carried out in the presence of canine microsomal membranes (Promega) (Hancock *et al.*, 1991).

Immunofluorescence/MDCK microinjection

MDCK cells were microinjected intracytoplasmically using a Zeiss/Eppendorf semi-automatic microinjecting device. Approximately 2×10^{-11} ml plasmid DNA at 0.2 mg/ml were injected. After 18−20 h at 37°C the cells were fixed in 3% paraformaldehyde/50 mM ammonium chloride/0.2% Triton X-100 for 10 min, washed and then incubated in monoclonal antibody 7F7 (Schultz *et al.*, 1988) at 1:1000 dilution in PBS-A for 1 h, followed by a 1:400 dilution of goat anti-mouse immunoglobulin coupled to FITC (Pierce). Preparation of NIH3T3 cell lines expressing transfected K-ras(B) proteins has been described previously (Hancock *et al.*, 1990). Cells were examined using an MRC 500 confocal imaging system in conjunction with a Nikon Optiphot fluorescent microscope with a ×60 planapo objective lens.

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