The EMBO Journal vol.3 no.11 pp.2581 – 2585, 1984

Harvey murine sarcoma virus p21 *ras* protein: biological and biochemical significance of the cysteine nearest the carboxy terminus

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Communicated by N.Fiil

Previous studies of premature chain termination mutants and in frame deletion mutants of the p21 *ras* transforming protein encoded by the transforming gene of Harvey murine sarcoma virus (Ha-MuSV) have suggested that the C terminus is required for cellular transformation, lipid binding, and membrane localization. We have now further characterized the post-translational processing of these mutants and have also studied two C-terminal v-*ras*^{H} point mutants: one encodes serine in place of cysteine-186, the other threonine for valine-187. The Thr-187 mutant was transformation-competent, and its p21 protein was processed normally, as was the p21 encoded by a transformation-competent deletion mutant from which amino acids 166 – 175 had been deleted. The Ser-186 mutant was defective for transformation. The p21s encoded by the Ser-186 mutant and by the previously described transformation-defective mutants did not undergo the post-translational processing common to biologically active *ras* proteins: their electrophoretic migration rate did not change, they remained in the cytosol, and they failed to bind lipid. Since the cell-encoded *ras* proteins also contain this cysteine, we conclude that this amino acid residue is required for all *ras* proteins.

Key words: ras genes/cellular transformation/mutagenesis/protein processing

Introduction

The p21 protein encoded by the Harvey murine sarcoma virus (Ha-MuSV) v-*ras*^{H} gene, which induces focal transformation of NIH 3T3 cells, is responsible for the high oncogenicity of this rat-derived acute transforming retrovirus (Ellis *et al.*, 1982). The p21 coding sequences of the viral transforming gene have been derived from the rat cellular proto-oncogene c-*ras*^{H}-1 (DeFeo *et al.*, 1981). The cellular and viral *ras*^{H} genes each encode a 189 amino acid protein product, with the primary amino acid sequence of v-*ras*^{H} differing from c-*ras*^{H}-1 only at residues 12 and 59 (Dhar *et al.*, 1982; Ruta, Scolnick and Dhar, personal communication). Activated forms of c-*ras* genes with point mutations in p21 coding sequences have been found in a wide variety of human and animal tumors (reviewed in Land *et al.*, 1983).

The mechanism by which p21 induces transformation is unclear. It is known that the primary translation product (pro p21) of either the viral or cellular gene, which is found initially in the cytosol, is subjected to post-translational modification (Shih *et al.*, 1982b; Papageorge *et al.*, 1982), binds lipid (Sefton *et al.*, 1982), and becomes localized to the inner surface of the plasma membrane (Willingham *et al.*, 1980). The change in sub-cellular location and the lipid binding are associated with a slightly faster electrophoretic migration rate of the protein in SDS-polyacrylamide gels. No hydrophobic leader or signal peptide has been identified at the N-terminus of pro p21. Rather, the change in migration rate associated with the post-translational processing has been localized to the C half of the protein, based on results of formic acid and V8 protease cleavage studies of the viral p21 (Shih *et al.*, 1982b). It has not yet been determined experimentally if this change in mobility is a consequence of the added lipid or might also be the result of additional, as yet unidentified modification(s), such as proteolytic cleavage of certain amino acids from the primary translation product.

In contrast to the pp60 *src* protein the *ras* proteins bind guanine nucleotides non-covalently *in vitro* and do not possess a detectable tyrosine kinase activity (Scolnick *et al.*, 1979; Papageorge *et al.*, 1982). The substitution in v-*ras*^{H} of threonine at amino acid 59 for the alanine encoded at this residue in c-*ras*^{H}-1 leads to the post-translational phosphorylation of this threonine in some p21 molecules (Shih *et al.*, 1982a).

Table I. C-terminal *ras* amino acids

Gene	Terminal amino acids						Biological activity
	184	185	186	187	188	189	
ras^{H}(c,v)	Cys	Lys	Cys	Val	Leu	Ser	Yes
ras^{N}(c)	Leu	Pro	Cys	Val	Val	Met	Yes
ras^{K}(c-4A)	Lys	Lys	Cys	Ile	Ile	Met	Yes
ras^{K}(c-4B)	Thr	Lys	Cys	Val	Ile	Met	Yes
ras^{K}(v)	Lys	Lys	Cys	Val	Ile	Met	Yes
Yeast *ras*$^{SC-1}$	Gly	Cys	Cys	Ile	Ile	Cys	Yes
Yeast *ras*$^{SC-2}$	Gly	Cys	Cys	Ile	Ile	Ser	Yes
pBW769	Cys	Lys	–	Leu	Ile	Ser	No
pBW277	Cys	Lys	Cys	Thr		Pro	No
pBW858	Cys	Lys	Ser	Val	Leu	Ser	No
pBW859	Cys	Lys	Cys	Val	Leu	Ser	Yes
pBW945	Cys	Lys	Cys	Thr	Leu	Ser	Yes

The six amino acids at the C terminus are shown for each gene (except for mutants pBW769 and 277, where the five terminal residues are listed). Most, but not all, *ras* genes encode 189 amino acids. The actual number of amino acids encoded by each gene are: 189 for c-*ras*^{H}, v-*ras*^{H}, c-*ras*^{N}, c-*ras*^{K}-4A and v-*ras*^{K}; 188 for c-*ras*^{K}-4B; 309 for yeast *ras*$^{SC-1}$ and 322 for yeast *ras*$^{SC-2}$. Mammalian c-*ras* sequence data are from human genes: c-*ras*^{H} (Capon *et al.*, 1983; Reddy, 1983), v-*ras*^{H} (Dhar *et al.*, 1982), c-*ras*^{N} (Taparowsky *et al.*, 1983), c-*ras*^{K} (Shimizu *et al.*, 1983; McGrath *et al.*, 1983), v-*ras*^{K} (Tsuchida *et al.*, 1982), c-*ras*$^{SC-1}$ and c-*ras*$^{SC-2}$ (Powers *et al.*, 1984; Dhar *et al.*, 1984). Biological activity of the mammalian *ras* genes has been demonstrated on NIH 3T3 cells (reviewed in Land *et al.*, 1983). Biological activity of the two yeast genes has been demonstrated by the effects of their deletion from *S. cerevisiae* (Tatchell *et al.*, 1984). Data for mutants pBW 769 and 277 are from Willumsen *et al.* (1984). Data for pBW858, 859 and 945 are from this paper.

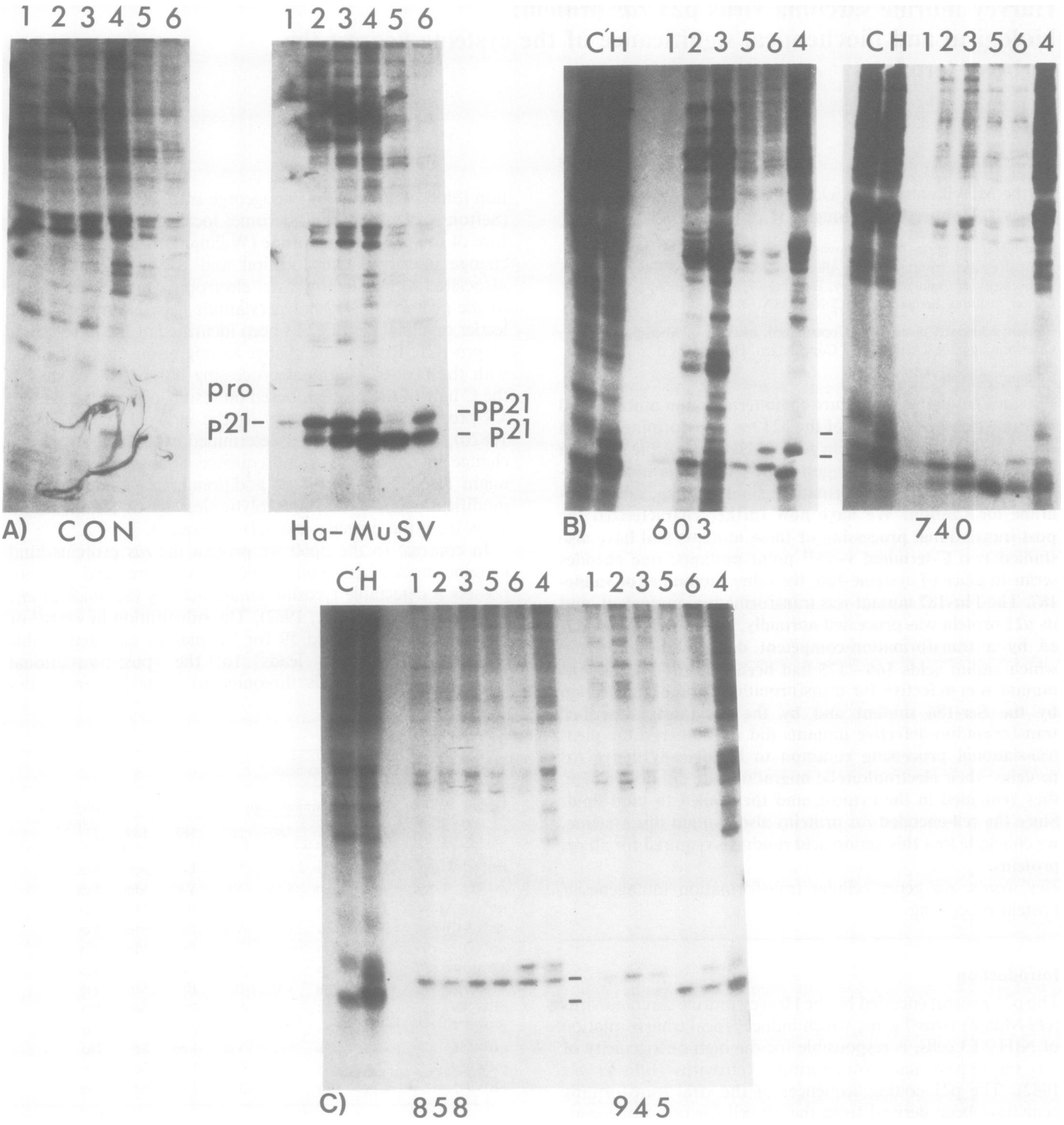


Fig. 1. Immunoprecipitation of mutant p21 proteins labelled under pulse-chase conditions. Cultures transfected with Ha-MuSV mutants were metabolically labelled with [^{35}S]methionine (250 μCi/ml) in methionine-free medium. Extracts of whole cells were prepared and precipitated with a p21 monoclonal antibody (Y13-238; Furth *et al.*, 1982) as previously described (Papageorge *et al.*, 1982). Immunoprecipitates were subjected to electrophoresis in 15% SDS-polyacrylamide gels, autoradiographed, and visualized after 48 h. **Lanes 1, 2, 3** and **4** refer to 10 min, 30 min, 60 min and 24 h pulses, respectively. **Lanes 5** and **6** refer to 1 h pulses followed by a 3 h and 24 h chase, respectively. **Lane C** = NIH 3T3 cells, **lane C'** = NIH 3T3 tk$^-$ cells, **lane H** = wild-type p21. In **panel A**, pro p21 indicates the primary *ras* translation product, p21 the mature non-phosphorylated form of the protein, and pp21 the phosphorylated form. The drawn lines in the center of **panels B** and **C** represent the migration rate of wild-type p21 and pp21. CON = tk$^-$ NIH 3T3 cells; Ha-MuSV = NIH 3T3 cells transformed by wild-type v-*ras*^{H} (labelling of tk$^-$ cells transfected with wild-type v-*ras*^{H} gave identical results); 603 = pBW603, a transformation-defective premature termination mutant encoding 184 amino acids (residues 1–183 are the same as wild-type, and 184 is proline); 740 = pBW740, a transformation-competent mutant in which amino acids 166–175 have been deleted (and replaced by Pro-Asp-Gln); 858 = pBW858, the transformation-defective Ser-186 mutant; 945 = pBW945, the transformation-competent Thr-187 mutant.

We have recently begun to assess the possible relevance of these post-translational features of the viral p21 protein to cellular transformation. Previous results obtained with v-*ras*^{H} encoded premature chain termination mutants and in frame deletion mutants suggested that one or more of the six C-terminal amino acids were required for three functions: (i) focal transformation of NIH 3T3 cells; (ii) membrane localization of the protein; and (iii) the binding of lipid to the protein. Since Cys-186 was uniquely conserved among the six C-terminal amino acids of all *ras* genes (Table I; Powers *et al.*, 1984; Dhar *et al.*, 1984), we speculated that this residue might be essential for these functions (Willumsen *et al.*, 1984). We have now tested this hypothesis by using site-directed mutagenesis to substitute Ser-186 for this cysteine residue. Our results indicate that this mutant protein is deficient for cellular transformation and for the post-translational modifications of pro p21, including membrane localization, lipid binding, and altered electrophoretic migration rate.

Results

Table I depicts the six C-terminal amino acids of different biologically active mammalian and yeast *ras* genes that have been sequenced. The cysteine (amino acid residue 186 for most mammalian *ras* genes, including v-*ras*^{H}) that is located three amino acids from the terminal amino acid is present in each gene. It is followed by two hydrophobic amino acids (valine, leucine, or isoleucine). One of the transformation-defective mutants of the previous study (pBW769 in Table I; Willumsen *et al.*, 1984) could be viewed as encoding a v-*ras*^{H} gene from which Cys-186 had been deleted (Table I). This experimental result made it likely that Cys-186 might be essential for p21 function. A second transformation-defective mutant (pBW277), which encoded Thr-Pro for residues 187 – 189, suggested that C-terminal amino acids in addition to Cys-186 might also be important for biological activity.

Serine-186 mutant lacks transforming activity

To test the possible importance of Cys-186 and Val-187, site-directed mutagenesis was employed to generate two point mutants in v-*ras*^{H}: one mutant encodes Ser-186 (clone pBW858) in place of Cys-186, the other Thr-187 (clone pBW945) for Val-187 (Table I). When the transforming activity of the mutant DNAs was tested on NIH 3T3 cells, the Ser-186 mutant failed to induce morphologic changes. The Thr-187 mutant and a reconstructed wild-type molecule (clone pBW859) whose coding squences encoded Cys-186 transformed the 3T3 cells with high efficiency ($>10^3$ foci/μg DNA).

Transformation-defective mutants do not process their pro p21 normally

The primary *ras* translation product is a cytosol-associated pro p21 which is processed to a faster migrating form (p21) that localizes to the plasma membrane and binds lipid. About one-quarter of the viral p21 molecules become phosphorylated at Thr-59; the phosphorylated form (pp21) has a slower migration rate so that the membrane-associated protein appears as a doublet in most gels (pp21 and p21; see Figure 1).

Since our earlier experiments with the premature termination and deletion mutants had not determined if their p21 proteins were processed in a similar manner, we studied this parameter for these mutants as well as for the two point mutants (Figure 1). To characterize the p21 proteins encoded

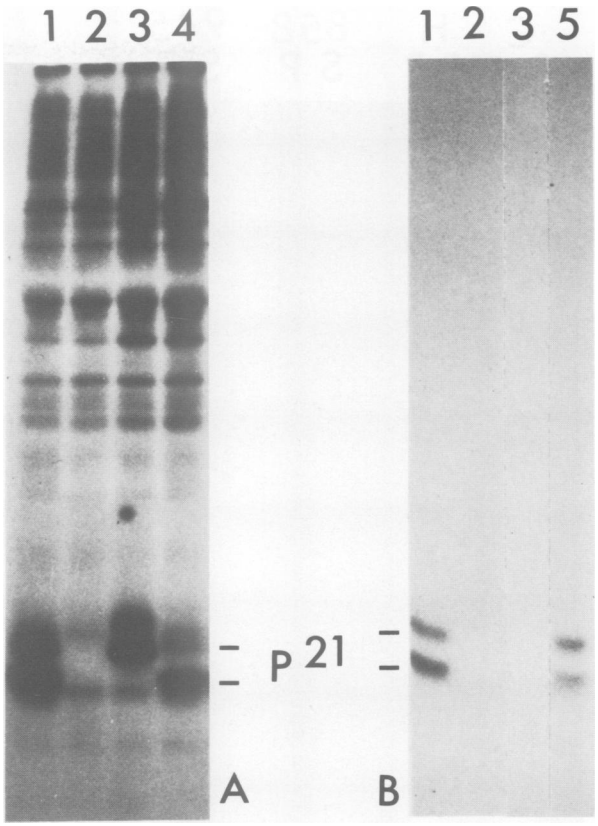


Fig. 2. Immunoprecipitation of methionine and lipid labelled v-*ras*^{H} point mutants. Cultures were metabolically labelled for 24 h with [^{35}S]methionine (250 μCi/ml) in **panel A** and with [^{3}H]palmitic acid (1 mCi/ml) in **panel B**. Immunoprecipitation with p21 monoclonal antibody Y13-238 and gel electrophoresis were performed as in Figure 1. Autoradiograms were exposed for 48 h. Extracts in **panels A** and **B** are: **lane 1**, wild-type Ha-MuSV DNA transfectant; **lane 2**, NIH 3T3 tk$^-$ cells; **lane 3**, pBW858 (Ser-186 mutant); **lane 4**, pBW859 (reconstructed wild-type p21); **lane 5**, pBW945 (Thr-187 mutant).

by the transformation-defective mutants, the DNAs (which also contained an intact HSV tk gene) were transfected into 3T3 cells that were tk$^-$ and selected for conversion to tk$^+$ by virtue of their ability to confer HAT resistance to the cells.

Expected results were obtained with the wild-type protein: a 10 min pulse showed the pro p21, 30 and 60 min pulses demonstrated a mixture of pro p21 and p21, a 1 h pulse and 3 h chase revealed only the p21 form, and the pp21 form was first seen after the 24 h chase (Figure 1). Similar results were obtained with the Thr-187 mutant (pBW945) and a previously described transformation-competent mutant (pBW740) from which amino acids 166 – 175 had been deleted.

By contrast, the pro p21 encoded by each type of C-terminus transformation-defective mutant [the Ser-186 mutant (pBW858), a premature termination mutant (pBW603) and a mutant from which amino acids 181 – 186 had been deleted (pBW755; data not shown)] was not chased to the faster migrating p21 form (Figure 1). The results indicate that these defective proteins do not process the pro p21 as do the biologically active proteins. After overnight labelling, a transformation-defective protein carrying a mutation at the C terminus will therefore migrate more slowly in gels than will the p21 of a transformation-competent gene encoding a similar number of amino acids (compare pBW858 and 859 in Figure 2A).

Although the defective proteins did not process their pro

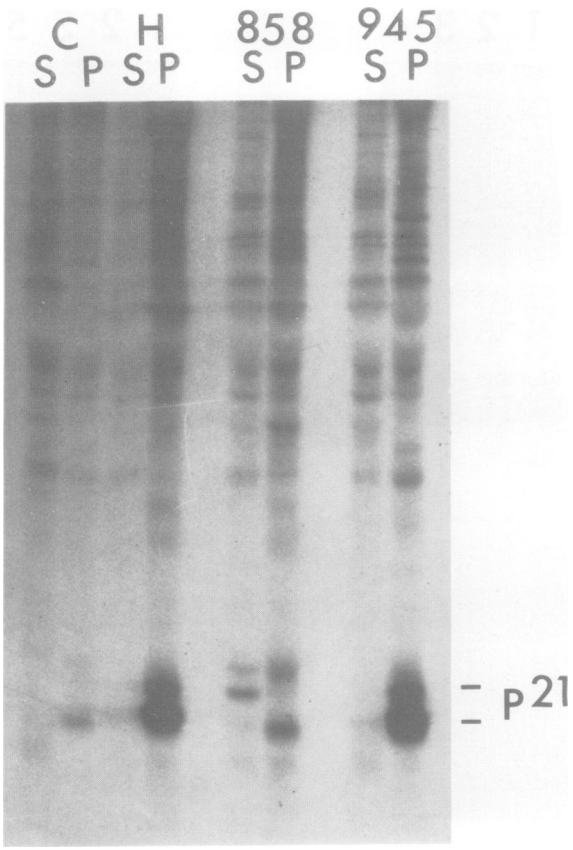


Fig. 3. Fractionation of methionine-labelled point mutants. Cultures containing control and mutant DNAs were metabolically labelled overnight with [^{35}S]methionine. Hypotonic swelling of the cells was followed by homogenization and low speed centrifugation to remove nuclei. The supernatant was then fractionated into a pellet particulate fraction containing the plasma membranes and a supernatant cytosol fraction as described (Courtneidge *et al.*, 1980; Shih *et al.*, 1982b; this procedure separates the cytosol-associated pro p21 from the membrane-associated mature p21 forms). The fractions were precipitated with monoclonal p21 antibody Y13-238, electrophoresed, and autoradiographed for 4 days. The lanes labelled S and P contain the supernatant-cytosol and pellet-membrane fractions, respectively. C = control tk$^-$ cells, H = wild-type Ha-MuSV DNA transfectant, 858 = pBW858 (Ser-186 mutant), 945 = pBW945 (Thr-187 mutant). Longer exposure did not reveal any p21 in the membrane fraction of 858.

p21 normally, a slower migrating form analogous to the phosphorylated form of competent p21 proteins was noted as in the wild-type protein (Figure 1, pBW603 and 858); metabolic labelling with ^{32}P indicated that this latter form represents a phosphorylated form of the pro p21 (pro pp21; data not shown). Since the p21 proteins encoded by the transformation-defective mutants do not localize to the membrane (see below; Willumsen *et al.*, 1984), the results suggest that phosphorylation of the protein can take place in the cytosol on a transformation-defective p21. The possible biological importance of the v-*ras*^{H} phosphorylation at Thr-59 therefore still remains to be established.

Ser-186 mutant is not membrane associated and does not bind lipid

We have previously found that, in contrast to the wild type *ras* protein, the *ras* protein encoded by the transformation-defective C-terminal premature termination and deletion mutants did not localize to the plasma membrane and failed to bind lipid. Similar results have now been obtained with the Ser-186 mutant (Figures 2 and 3).

The protein encoded by Ser-186 protein did not label with [^{3}H]palmitic acid under conditions where the processed, membrane-associated forms of the wild-type protein were labelled (Figure 2B). These results suggested that the Ser-186 protein was not bound to the plasma membrane. To confirm this prediction, metabolically labelled cell extracts were divided into crude supernatant-cytosol (S) and pellet-membrane (P) fractions (Figure 3); >90% of the wild-type protein and the Thr-187 mutant p21 (pBW945) was associated with the membrane fraction, while all the Ser-186 encoded protein was found in the cytosol.

Discussion

Our earlier studies of premature termination and in frame deletion mutants had suggested that one or more of the six C-terminal amino acids were required for the biological activity of p21 as well as for its membrane localization and lipid binding (Willumsen *et al.*, 1984). The close correlation between cell transformation and these biochemical features of the protein suggested that to be biologically active the protein must bind lipid and be at the plasma membrane. The current studies emphasize the critical importance of Cys-186 to the capacity of p21 to transform cells; the data also link the post-translational processing of pro p21 to the lipid binding and membrane localization of the mature protein. A mutant p21 encoding Ser-186 is defective for transformation and the post-translational changes, although this mutation only substitutes an oxygen atom (serine) for a sulfur atom (cysteine). These results suggest that Cys-186 is directly involved in the post-translational events leading to the biologically active membrane-associated form of p21. Since cysteine occupies a similar location in all known *ras* genes (Table I), we speculate that this cysteine subserves a similar essential function in other *ras* genes.

The premature termination mutants, C-terminal deletion mutants, and point mutants we have characterized can thus be divided into two phenotypes. The wild-type pro p21 and those encoded by transformation-competent C-terminal mutants undergo post-translational processing that involves a change in electrophoretic migration rate in association with the binding of lipid to the protein and its localization to the plasma membrane. By contrast, the pro p21 protein encoded by the Cys-186 mutant and the other transformation-defective C-terminal mutants remains associated with the cytosol, does not bind lipid, and does not change its migration rate. These results suggest that the properties of p21 that lead to the change in migration rate represent an integral part of the process leading to lipid binding and membrane association of the protein.

While these data establish the importance of Cys-186 for the p21, they do not determine how this amino acid functions in the protein. The cysteine might form a required intramolecular disulfide bridge with another cysteine in p21, but this cysteine nearest the C terminus is the only cysteine that is conserved among all mammalian and yeast *ras* genes (see Powers *et al.*, 1984). The cysteine might form an intermolecular disulfide linkage with another polypeptide, but immunoprecipitation under non-reducing conditions does not suggest that such linkage exists (Finkel and Cooper, 1984; M.O.Weeks and E.M.Scolnick, personal communication). Although the cysteine may merely place the protein in a conformation required for biological activity, we speculate that the lipid may be bound to the p21 through the cysteine. Such

binding may be via a thio-ether linkage to diacylglycerol, as has been found for membrane-associated murein-lipoprotein of *Escherichia coli* (Hantke and Braun, 1973), or as a thiol ester; as has been suggested by Bolanowski *et al.* (1984), based on their studies of palmitic acid labelling of proteins in sea urchin embryos. The release of palmitic acid label from p21 with hydroxylamine indicates that the fatty acid is not bound in an amide linkage to the protein (Sefton *et al.*, 1982).

Cys-186 is followed by two hydrophobic amino acids (valine, leucine or isoleucine) in each *ras* gene. Substituting these and the terminal serine with threonine-proline (pBW277, Table I) confers the same phenotype as the Ser-186 mutant: transformation defectiveness with loss of processing, lipid binding and membrane association. The requirement for a hydrophobic domain close to the Cys-186 is, however, not absolute, since the substitution of threonine for Val-187 results in a protein that apparently transforms cells as efficiently as the wild-type protein. Further analysis might reveal subtle biological differences in the Thr-187 mutant.

It is still not clear whether the post-translational change in gel migration rate is due to proteolytic cleavage, lipid binding, or to other changes in the molecule. Previous studies have indicated that the N terminus is not involved in this modification (Shih *et al.*, 1982b). It has been suggested that the maturation of the protein is associated with cleavage of pro p21 at amino acids 167–170 via a membrane peptidase (Shimizu *et al.*, 1983). Our experimental results argue strongly against this possibility. Mutants from which the coding region for these amino acids have been deleted can retain their transforming activity and the protein encoded by such mutants is processed as is the wild-type protein. The importance of Cys-186 makes it likely that no more than three amino acids are cleaved during processing.

Materials and methods

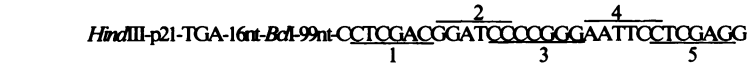
Cells and DNA-mediated gene transfer

The NIH 3T3 cells, tk$^-$ NIH 3T3 cells, and DNA transfection procedure have been previously described (Willumsen *et al.*, 1984). Cells were grown in Dulbecco's modified MEM supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml). The tk$^-$ cells were maintained on 100 μg/ml BrdUrd until used. Transfection of DNA was carried out with calcium (Graham and van der Eb, 1973), using 25 μg/ml NIH 3T3 DNA as carrier. Duplicate dishes were transfected with 0.2 μg of DNA, and foci were counted 11–14 days later. Transfection of the plasmid DNA to tk$^-$ cells was selected in HAT medium.

Plasmid DNA and site-directed mutagenesis

The starting clone pBW276 has been described (Willumsen *et al.*, 1984). It consists of the transforming region of Ha-MuSV DNA (the viral long terminal repeat and p21 coding sequences), the *neo*^{R} gene from Tn5 (for selection in *E. coli*), the Herpes simplex virus (HSV) *tk* gene (for selection of transformation-defective p21 genes in tk$^-$ cells), and the pBR322 origin of replication. The clone contains a *Hind*III site located at amino acid 5 in the coding region of p21 as well as a unique *Xho*I site located 115 nucleotides downstream from the p21 TGA stop codon.

The Ser-186 mutant was constructed by a previously described procedure (Norris *et al.*, 1983). First, a single-stranded m13 phage (Messing and Vieira, 1982) mpBW828 was constructed (derived from phage mp9) carrying p21 sequences on a modified *Hind*III/*Xho*I fragment with the structure:



(1 = *Xho*I/*Sal*I, 2 = *Bam*HI, 3 = *Sma*I, 4 = *Eco*RI, 5 = *Xho*I).

A 17-mer oligonucleotide that was complementary to v-*ras*^{H} nucleotides 889-905 (amino acids 183–188; numbering of sequences is according to Dhar *et al.*, 1982) except for residue 896, which contained an equal mixture of T and C residues, was then annealed to mpBW828 along with the normal sequencing primer and replicated *in vitro*. The 17-mer should therefore change the codon of Cys-186 from CGT to AGT (ser) and to GGT (Gly) in some molecules. After phenol extraction, the DNA was cut with *Hind*III and *Xho*I and ligated to an m13 fragment (a derivative of mp9) containing *Hind*III and *Xho*I ends. A mutant DNA (clone pBW845) encoding AGT (Ser) was selected by hybridization with the oligonucleotide. No mutant carrying GGT was isolated. A full-length plasmid analogous to pBW276 was then constructed from the mutant *Hind*III/*Xho*I fragment of pBW845 or from the wild-type *Hind*III/*Xho*I fragment of pBW828. Clone pBW858 is the full-length Ser-186 mutant clone, while clone pBW859 is the reconstructed wild-type clone. Comparative dideoxysequencing (Sanger *et al.*, 1977) of the entire p21 coding region of pBW858 and pBW859 revealed no coding differences except for the C to A transversion at nucleotide 896 changing Cys to Ser. The mutant containing Thr-187 in place of Val-187 was constructed in a similar manner, using a 24-mer oligonucleotide complementary to nucleotides 888–911 that converted the wild type GTG-187 (valine) to ACG (threonine). The full length reconstructed Thr-187 mutant is clone pBW945.

Acknowledgements

We thank Niels Fiil and Henry Krutzsch for helpful discussions and Annette Christensen and Marilyn Lander for expert technical assistance. B.M.W. has been a Senior Fellow at the University of Copenhagen.

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Received on 13 July 1984; revised on 31 July 1984