Journal of Cellular Biochemistry 61:172–181 (1996)

Normal and Transforming Ras Are Differently Regulated for Posttranslational Modifications

Toshiko Yamada-Okabe, Rikuo Doi, and Hisafumi Yamada-Okabe

Department of Hygiene, Yokohama City University, School of Medicine 3-9, Fukuura, Kanazawa-ku, Yokohama 236, Japan

Abstract Point mutation of the c-H-*ras* gene significantly increases cellular transforming activities of Ras. Since posttranslational modification and subsequent membrane localization are essential for the biological activities of Ras, we examined whether or not the mutation also affects these two factors. The normal (Gly^{12}) or the transforming (Val^{12}) c-H-*ras* gene was expressed in NIH3T3 cells using a metallothionein promoter. Expression of either type of Ras was efficiently induced by the cadmium treatment of these cells, and immunoprecipitation of metabolically labeled cell extracts revealed that both normal and transforming Ras were expressed as four differently migrating forms on SDS-polyacrylamide gels, two of which were slower migrating cytosolic precursors and the other two were faster migrating membrane-bound forms. There was no significant difference in half lives between normal and transforming Ras; however, posttranslational modification was quite different between the two types of Ras. Transforming Ras was processed and became membrane-bound forms much more efficiently than normal Ras. Interestingly, posttranslational modification and membrane localization of Ras was significantly inhibited when the c-*myc* oncogene was co-expressed with Ras. In contrast to the c-*myc* oncogene, expression of either wild type or mutant p53 did not affect the posttranslational modification of Ras, suggesting that the c-*myc* oncogene specifically impairs the posttranslational modification of Ras.

Key words: c-H-*ras*, Ras, posttranslational modification, NIH3T3, c-*myc*, p53

The *ras* genes encode 21-kD proteins (Ras) that bind to GTP or GDP and affect cell proliferation and differentiation. Point mutation may convert the ras genes into oncogenes [Lowy and Willumsen, 1993; Weinberg, 1989]. The presence of the ras oncogenes in a large variety of human tumors [Weinberg, 1989] suggests that this conversion may be a critical step in tumor development [Weinberg, 1989].

Ras is modificated at posttranslational level, and this posttranslational modification is essential for the membrane localization and biological activities of Ras [Willumsen et al., 1984]. Posttranslational modification of Ras involves (1) cleavage of C-terminal 3-amino acids, (2) farnesylation of cysteine at the newly produced C-terminal end, (3) palmitoylation of other cysteine located near the C-terminal end, and (4) phosphorylation. Cleavage of C-terminal 3-amino acids and farnesylation of cysteine are essential for the membrane localization of Ras, but subsequent palmitoylation and phosphorylation are not necessary for the activities [Hancock et al., 1989].

Once Ras becomes a membrane-bound form, activated tyrosine kinase type receptors transmit factors as Grb2 and Sos to signal Ras [Buday and Downward, 1993; Chardin et al., 1993; Egan et al., 1993; Gale et al., 1993; Li et al., 1993; Oliver et al., 1993; Rozakis-Adcock et al., 1993; Simon et al., 1993]. The activated Ras interacts with the cytoplasmic serine, threonine kinase, Raf [Moodie et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993], and increases the phosphorylation (predominantly on serine residues), as well as membrane recruitment of Raf [Stokoe et al., 1994]. The activated Raf may phosphorylate and activate several transcription factors including Fos and Jun through the MAP kinase cascade. The other signaling pathway from Ras involves the association and

Abbreviations used: SDS, sodium dodecyl sulfate; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonylfluoride.

Received August 11, 1995; accepted October 2, 1995.

Address reprint requests to Dr. Hisafumi Yamada-Okabe, Department of Hygiene, Yokohama City University, School of Medicine, 3-9, Fukuura, Kanazawa-ku, Yokohama 236, Japan.

activation of phosphatidyl inositol 3 kinase that may also lead to the activation of many genes [Rodriguez-Viciana et al., 1994].

On the other hand, tumor progression is the consequence of a series of genetic alterations including the activation of protooncogenes and the inactivation of tumor suppresser genes [Weinberg, 1989]. In vitro, the *ras* oncogenes can cooperate with the myc, mutant p53, adenovirus E1A and polyoma virus T oncogenes [Land et al., 1983; Ruley, 1983]. Thus, the *ras* oncogene alone cannot transform primary cells in culture, whereas together with the c-*myc* (or some other) oncogene, it can [Land et al., 1983]. Such a cooperative transforming ability of the *myc* and the *ras* oncogenes was observed also in vivo; the mating of transgenic mice bearing a c-*myc* oncogene with the *ras* oncogene gives rise to offspring with a synergistic increase in tumor formation [Sinn et al., 1987]. Furthermore, the *ras* oncogene-induced hyperplasia is often associated with the expression of mutant p53, whereas a combination of the *ras* and *myc* oncogenes can induce malignancy even in the presence of a wild-type p53 [Lu et al., 1992].

Here we present the data indicating that normal and transforming Ras undergo different posttranslational modifications. Furthermore, the c-*myc* gene, when co-expressed with the c-H-*ras*, negatively regulates the posttranslational modification of Ras, whereas mutant p53 does not. This negative regulation might be one of the mechanisms of cross-talk between the *ras* and the c-*myc* oncogene.

MATERIALS AND METHODS

Plasmids

pMTcHr(G^{12}) and pMTcHr(V^{12}) which carry normal (Gly^{12}) and transforming (Val^{12}) c-H-*ras* cDNA, respectively, linked to a metallothionein promoter, were generous gifts from Dr. S. Nakamura and Dr. S. Hattori (National Institute of Neurosciences, Japan). pCDM8-p53W and pCDM8-p53M, harboring wild type and mutant human p53 cDNA together with G418 resistant gene, respectively, were from Dr. K. Morishita (National Cancer Institute, Japan). pFRSV, carrying an altered form of dihydrofolate reductase cDNA [Simonsen and Levinson, 1983], was donated by Dr. A. Horwich (Yale University). fpGV-*myc*, a retrovirus vector carrying the murine c-*myc* and the neomycin resistance gene [Dean et al., 1987], was provided by Dr. J.L. Cleveland (St. Jude Children's Research Hospital). pSV2-γ-actin encoding human γ-actin cDNA was a gift from Dr. Y. Dobashi (University of Tokyo).

Cells and DNA Transfection

Unless otherwise specified, monolayer cultures of NIH3T3 and psi3 were maintained in DMEM supplemented with 10% calf serum. Transfection of DNA into these cells was carried out by the calcium phosphate gel precipitation method with a glycerol shock [Davis et al., 1986]. Subconfluent cultures of the cell lines were transfected with the DNA indicated. 24 h after transfection, 5×10^5 cells were seeded on 10 cm dishes. At that time, a selective drug was added to the cell culture, and the selection was carried out for 2 weeks:

1. *Transfection of myc:* To prepare recombinant retrovirus carrying c-*myc*, psi3 cells on 3.5-cm dishes were transfected with 5 μg fpGV-*myc* DNA. Selection was performed using 1 mg/ml G418 (Gibco-BRL, Grand Island, NY), and several G418-resistant colonies were isolated with cloning cylinders.
2. *Transfection of ras:* subconfluent NIH3T3 cells on 10-cm dishes were transfected with 25 μg of pMTcHr(G^{12}) or pMTcHr(V^{12}) DNA together with 1 μg of pFRSV DNA. The selection was performed using 500 nM methotrexate (MTX) (Sigma, St. Louis, MO), and several MTX-resistant colonies were isolated.
3. *Transfection of p53:* Subconfluent cultures of NIH3T3 transfected with pMTcHr(G^{12}) or pMTcHr(V^{12}) in 3.5-cm dishes were further transfected with 1 μg of pCDM8–p53W or pCDM8-p53M. The selection was carried out using 1 mg/ml G418 for 2 weeks, and several hundred G418-resistant colonies were combined and used for further experiments.

Virus Preparation and Infection

The media from confluent cultures of isolated clones of psi3 cells that had been transfected with fpGV-*myc* (selected with G418, and found to produce high levels of the recombinant retrovirus) were replaced by fresh media. After further incubation for 24 h, the media were collected, filtered through a 0.45-μm filter, and stored at −80°C as virus solution. Subconfluent cultures of NIH3T3 which had been transfected with pMTcHr(G^{12}) or pMTcHr(V^{12}) in 3.5-cm

dishes were incubated with 1 ml of virus solution in the presence of 20 μg/ml polybrene (Sigma) for 2 h; 24-h postinfection, 5×10^5 cells were seeded on each 10-cm dish and further incubated for 24 h before adding G418 (1 mg/ml final concentration). The selection with G418 was carried out for 2 weeks, and several hundred G418-resistant colonies were combined and used for the experiments.

To induce Ras expression, NIH3T3 cells transfected with pMTcHr(G^{12}) or pMTcHr(V^{12}) were incubated in a medium containing $CdCl_2$ [10 μM for pMTcHr(G^{12}) or 2 μM for pMTcHr(V^{12})] for 24 h.

Northern Blotting

Cytoplasmic RNA was isolated from cell extracts treated with proteinase K and phenol, fractionated by agarose gel electrophoresis, transferred to nylon membranes, hybridized with labeled probes and visualized by autoradiography [Sambrook et al., 1989]; 4.8-kb *Xba*I/*Bam*HI fragment of pSV2-c-*myc* (for *myc*), 2.6-kb *Sac*I fragment of EJ 6.6 (for *ras*), and 1.0-kb *Pst*I/*Xba*I fragment of pSV2-γ-actin (for actin) were radiolabeled by the random priming method with α-[^{32}P]-dCTP and used as probes [Sambrook et al., 1989]. Hybridization and washing the filters were carried out under stringent conditions (50% formamide at 42°C for hybridization and 0.1 × SSC at 60°C for washing) [Sambrook et al., 1989].

Metabolic Labeling of the Cells

Subconfluent cultures of cells in 3.5-cm dishes were metabolically labeled with 200 μCi of [^{35}S]methionine/cysteine (spec act >1,000 Ci/mmole) for the indicated time, washed twice with PBS, and lysed in RIPA buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 1 mM PMSF, by passing through 21-gauge needles 10 times and then through 27-gauge needles 10 times. After removing cell debris by centrifugation at 12,000*g* for 15 min, supernatants were used as total cell extracts.

In order to examine the membrane localization of Ras, subconfluent cultures of cells in 6 cm dishes were metabolically labeled with 400 μCi of [^{35}S]methionine/cysteine (specific activity >1,000 Ci/mmole) for 1 h, washed with PBS, and lysed according to the method of Shih et al. [1982]. Cell extracts were then centrifuged at 100,000*g* for 60 min, and supernatants were used as S-100 cytosolic fractions. The precipitates were suspended in RIPA buffer and used as P100 membrane fractions after removing the debris by the centrifugation at 100,000*g* for 30 min.

For pulse-chase experiments, subconfluent cultures of cells in 3.5-cm dishes were metabolically labeled with 200 μCi of [^{35}S]methionine/cysteine (spec act >1,000 Ci/mmole) for 30 min, washed with DMEM twice to remove extra radiolabeled amino acids and incubated for the indicated time in DMEM supplemented with 10% FCS. After cells were washed twice with PBS, they were lysed in RIPA buffer as described earlier in this section.

Immunoprecipitation of Ras and p53

Ras was immunoprecipitated from the total cell extracts, S-100 fractions, and P-100 fractions with 2 μg/ml anti-Ras monoclonal antibody (Y13-259), together with 40 μg/ml antirat IgG and 30 μl of a 50% suspension of protein A sepharose CL-4B at 4°C overnight. The resulting immune complexes were washed twice with a buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 0.5% NP-40 and thereafter twice with a buffer containing 0.5 M LiCl and 100 mM Tris–HCl (pH 7.5) and 1% β-mercaptoethanol. Immunoprecipitation of p53 was the same as that of the Ras except for the use of anti-p53 antibody (PAb1801, 4 μg/ml) instead of anti-Ras monoclonal antibody. The immunoprecipitates were fractionated on 12.5% SDS-polyacrylamide gels (for Ras) or 10% SDS-polyacrylamide gels (for p53) and visualized by fluorography. Fluorography was carried out by soaking the acrylamide gels in 2 M salicylic acid solution for 30 min before drying. The anti-Ras monoclonal antibody (Y13-259) was purchased from Oncogene Science (Manhasset, NY) and anti-p53 monoclonal antibody (PAb1801) was obtained from Pharmingen (San Diego, CA).

RESULTS

At least some of the biological activities of the c-H-*ras* oncogene are much more pronounced than those of the H-*ras* protooncogene. Thus, microinjection of small amounts of Ras oncoprotein can induce DNA synthesis in quiescent cells, oocyte maturation, cell differentiation, and transformation, whereas larger amounts of normal Ras are needed to elicit such effects [Lowy and Willumsen, 1993]. Because membrane localization of Ras is essential for both types of Ras

proteins, we examined whether the posttranslational modification is differently regulated in normal (G^{12}) and transforming (V^{12}) Ras. For this purpose, we generated NIH3T3 cell lines in which the normal (G^{12}) or transforming (V^{12}) c-H-*ras* cDNA was expressed under the control of metallothionein promoter. The metallothionein promoter was chosen since it can be activated by $CdCl_2$ resulting in an about 20-fold increase in the level of both wild type (G^{12}) and mutant (V^{12}) c-H-*ras* mRNA (Fig. 1A). There was no difference in the levels of metal-induced normal and mutant ras mRNA, demonstrating that both types of the c-H-*ras* genes were equally expressed by metallothionein promoter.

Next we analyzed the posttranslational modification of Ras by metabolically labeling the above cells with [^{35}S]methionine/cysteine and by immunoprecipitating Ras with anti-Ras monoclonal antibody. In both NIH3T3 lines that harbored pMTcHr(G^{12}) or pMTcHr(V^{12}), four forms of differently migrating Ras proteins were detected by SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 1B). Fractionation of the cell extracts revealed that the two faster migrating Ras were in P-100 membrane fractions and the two slower migrating ones were in S-100 cytosolic fractions, demonstrating that the former two were membrane-bound forms and the latter two were non-farnesylated precursors. This is consistent with the results reported by Hancock et al. [1989] that faster migrating forms were farnesylated (some of which were further palmitoylated), and that slower migrating Ras were

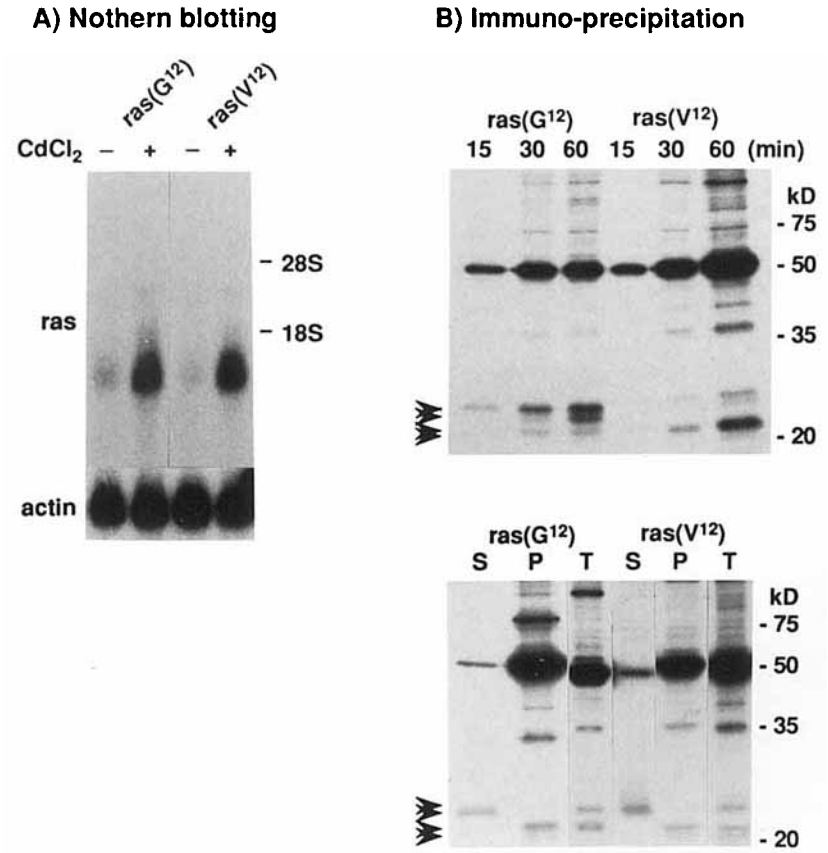

Fig. 1. Northern blotting of the *ras* mRNA (**A**) and immunoprecipitation of Ras (**B**) in NIH3T3 cells harboring pMTcHr(G^{12}) or pMTcHr(V^{12}). **A:** Cells were cultured in the presence or absence of $CdCl_2$ for 24 h. Twenty five μg of total cytoplasmic RNA extracted from these cells was fractionated on an agarose gel, and analyzed by Northern blotting, using the *ras* and actin probes. The positions of 28S and 18S ribosomal RNA are indicated. **B:** Cells were cultured in a medium containing $CdCl_2$ for 24 h, labeled with [^{35}S]methionine/cysteine for the indicated time, and 1.5-mg proteins of the total cell extracts were immunoprecipitated with anti-Ras monoclonal antibody. For the fractionation of cell extracts, cells were labeled with [^{35}S]methionine/cysteine for 30 min, and the total cell extracts were centrifuged prior to immunoprecipitation. The immune complexes were separated on a 12.5% SDS–polyacrylamide gel and Ras was visualized by fluorography. S, P, and T indicated S-100 fraction, P-100 fraction and total cell extracts, respectively. *Arrowheads,* positions of Ras. Positions of the marker proteins were also indicated in kD. For further details, see Materials and Methods.

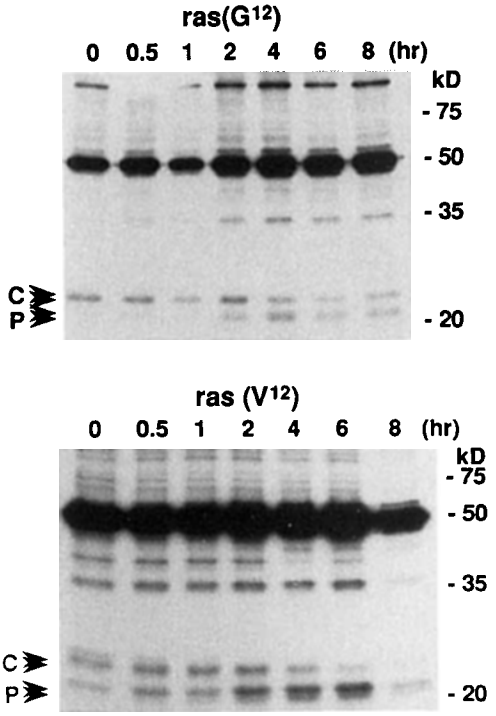

Fig. 2. Pulse-chase labeling of Ras in NIH3T3 cells harboring pMTcHr(G^{12}) or pMTcHr(V^{12}). Cells were cultured in a medium containing $CdCl_2$ for 24 h, labeled with [^{35}S]methionine/cysteine for 30 min, washed, and continued to culture for the indicated time in the presence of large excess of nonradiolabeled amino acids; 1.5 mg of the total cell extracts was immunoprecipitated with anti-Ras monoclonal antibody, and the immune complexes were separated on a 12.5% SDS–polyacrylamide gel. *Arrowheads P and C,* membrane-bound Ras and the cytoplasmic precursor of Ras, respectively. Positions of the marker proteins were also indicated in kD. For further details, see Materials and Methods.

unprocessed precursors. In fact, two of the faster migrating Ras were labeled with [^{14}C]mevalonic acid, whereas the other two were not (data not shown). Interestingly, most of Ras(G^{12}) were detected as slower migrating cytosolic precursors, but more than 80% of Ras(V^{12}) were faster migrating membrane-bound forms. There was a major band of 50 kD in the Ras immunoprecipitates. Since we also detected the same band even when the cell were not treated with $CdCl_2$ to induce Ras expression (data not shown), this might be the consequence of nonspecific binding of the protein to the antibody.

The different distribution of normal and transforming Ras in membrane and cytosolic fractions might raise the possibility that transforming Ras was modificated to be a membrane-bound form much faster than the normal Ras. To address this possibility, we carried out the pulse-chase labelling of both normal and transforming Ras and compared the conversion of precursors into membrane-bound forms between the two types of Ras. As shown in Figure 2, the faster migrating membrane-bound forms of Ras(G^{12}) were only detectable 2 h after chasing whereas about half of Ras(V^{12}) already became membrane-bound forms at 30 min after chasing, and at 2 hr after chasing, more than 80% of Ras(V^{12}) converted into faster migrating mature forms. Both normal and transforming Ras were long-lived proteins, and there was no significant difference in half-lives of proteins between the two types of Ras. Thus, it is obvious that transforming Ras was more efficiently converted into the faster migrating membrane-bound forms than normal Ras.

As noted, the c-*myc* oncogene was shown to cooperate with the ras oncogene in transformation, both in vitro and in vivo [Land et al., 1983; Sinn et al., 1987]. This prompted us to test whether the c-*myc* oncogene expression modulates the posttranslational modification of Ras. For this purpose, we infected NIH3T3 cells harboring pMTcHr(G^{12}) or pMTcHr(V^{12}) with the recombinant retrovirus fpGV-*myc* which expressed the c-*myc* DNA under the control of an LTR promoter to drive c-*Myc* expression, since this is a strong promoter. Northern blot analysis of c-*myc* RNA in the above infected cells revealed two major bands (Fig. 3). According to

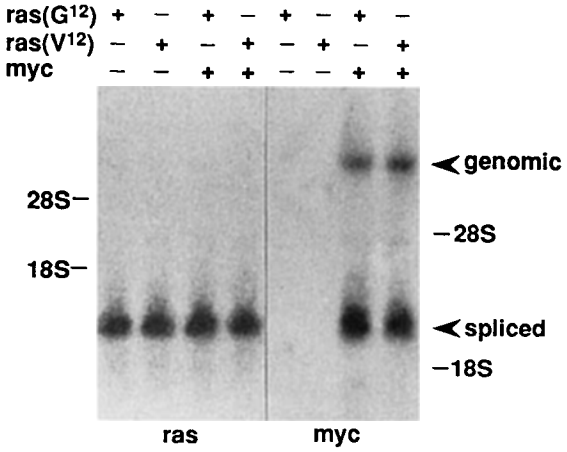

Fig. 3. Northern blotting of the c-*myc* mRNA in NIH3T3 cells harboring pMTcHr(G^{12}) or pMTcHr(V^{12}). Cells that had been infected or not infected with retrovirus carrying the c-*myc* oncogene were cultured in a medium containing $CdCl_2$ for 24 h. Twenty-five μg of total cytoplasmic RNA isolated from these cells was separated on a agarose gel, and analysed by Northern blotting using the *ras*- and *myc*-specific probes. The positions of 28S and 18S ribosomal RNA were indicated. For further details, see Materials and Methods.

Roussel et al. [1991], the slower migrating band corresponded to unspliced genomic RNA and the faster band to spliced *myc* mRNA. Furthermore, the levels of these c-*myc* specific RNAs were unaffected by metal-induced expression of either the normal or transforming c-H-*ras* (Fig. 3). Unless cells were infected with the virus carrying the c-*myc* oncogene, both normal and transforming Ras were expressed as four differently migrating forms, as demonstrated earlier (Figs. 1B, 4A). However, the c-*myc* oncogene expression diminished the two slower migrating forms of both normal and transforming Ras (Fig. 4A). Pulse-chase experiments showed that most of the normal as well as transforming Ras remained as slower migrating cytosolic precursors at least up to 8 h after chasing when the c-*myc* oncogene was constitutively expressed (Fig. 4B), whereas about half of the normal Ras and more than 90% of transforming Ras were already faster migrating membrane-bound forms by 6 h without infection with virus carrying the c-*myc* oncogene (Fig. 2). Thus, it becomes evident that the c-*myc* oncogene impairs the conversion of cytosolic precursor of Ras into the membrane-bound mature form. Although one additional slower migrating band of Ras(G^{12}) appeared at 4 h after chasing when the c-*myc* oncogene was expressed (Fig. 4B), its state of modification remains to be established.

Impairment of the posttranslational modification of Ras by the c-*myc* oncogene also prompted us to examine the possibility that other oncogenes that co-operate with the *ras* oncogene also

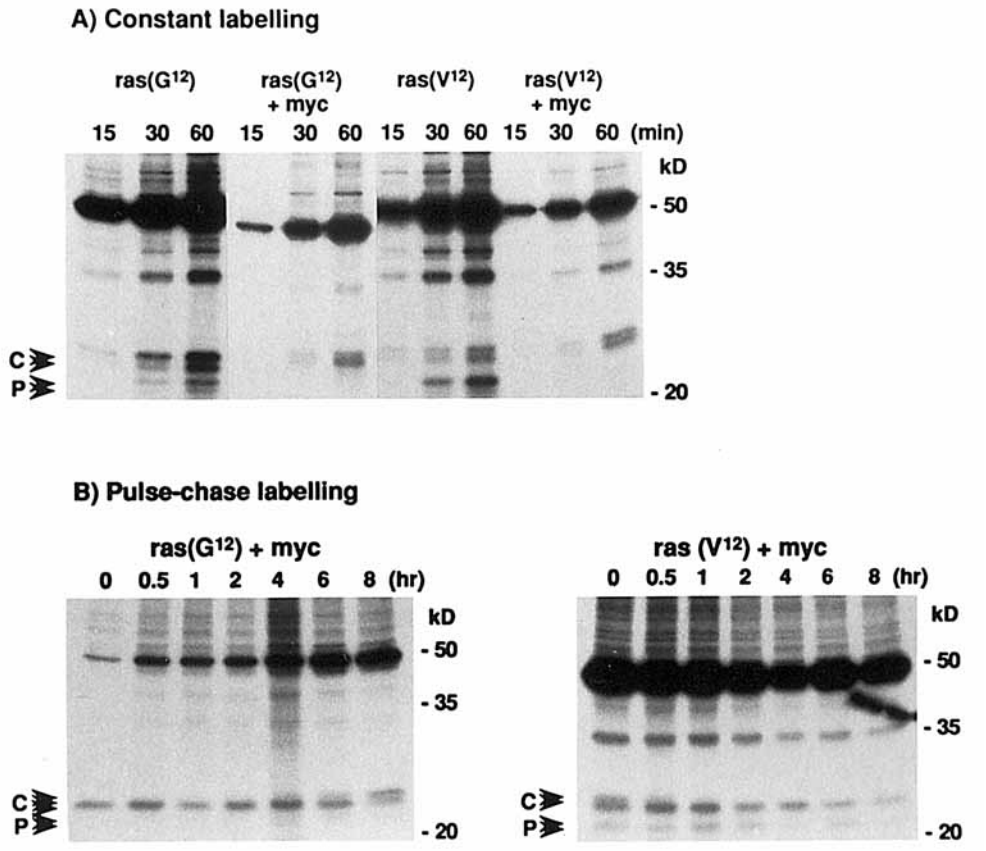

Fig. 4. Effects of the c-*myc* oncogene expression on the posttranslational modification of Ras in NIH3T3 cells harboring pMTcHr(G^{12}) or pMTcHr(V^{12}). **A:** Cells that had been infected or not infected with retrovirus carrying the c-*myc* oncogene were cultured in a medium containing $CdCl_2$ for 24 h, labeled with [^{35}S]methionine/cysteine for the indicated time; 1.5 mg of the total cell extracts were immunoprecipitated with anti-Ras monoclonal antibody, and the immune complexes were separated on a 12.5% SDS–polyacrylamide gel. **B:** Cells that had been infected with retrovirus carrying the c-*myc* oncogene were cultured in a medium containing $CdCl_2$ for 24 h, labeled with [^{35}S]methionine/cysteine for 30 min, washed, and continued to culture for the indicated time in the presence of large excess of nonradiolabeled amino acids; 1.5 mg of the total cell extracts was immunoprecipitated with anti-Ras monoclonal antibody, and the immune complexes were separated on a 12.5% SDS–polyacrylamide gel. *Arrowheads P and C,* membrane-bound Ras and the cytoplasmic precursor of Ras, respectively. Positions of the marker proteins were also indicated in kD. For further details, see Materials and Methods.

inhibit this effect. The p53 was chosen because, like the *ras* gene, point mutation of p53 converts it into an oncogene, and mutant p53 was found in a wide variety of human tumors [Hollstein et al., 1991] with an ability to cooperate with the c-H-*ras* oncogene in transformation and tumor development [Lane et al., 1983; Lu et al., 1992; Sinn et al., 1987]. The mutant p53 used in this study harbored the amino acid substitution of His for Arg at position 273 [Hollstein et al., 1991]. Immunoprecipitation of p53 proteins with anti-p53 monoclonal antibody revealed that there was no detectable level of the endogenous p53 in NIH3T3 cells and that either wild type or mutant p53 was efficiently expressed by the cytomegalovirus promoter (Fig. 5). Furthermore, neither normal nor transforming Ras affected the level of p53 expression (Fig. 5). Using these cells that constitutively expressed wild type or mutant p53 protein, effects of p53 on the posttranslational modification of Ras was examined by pulse-chase labeling of Ras proteins. Both normal and transforming Ras were fully expressed by $CdCl_2$ treatment even in the presence of wild or mutant p53 (Fig. 6). Unlike the case with c-*myc* oncogene, about 50% of normal Ras and about 90% of the transforming Ras were already membrane-bound forms even at 2 h after chasing (Fig. 6). As seen in the cells that were not transfected with the p53 cDNA but expressed Ras, conversion of slower migrating cytosolic precursors into faster-migrating membrane-bound forms occurred much more efficiently in the transforming Ras than in the normal. Thus, posttranslational modification of Ras was not significantly affected by the expression of either type of p53 (Fig. 6), and impairment of posttranslational modification of Ras by the c-*myc* oncogene was rather a specific effect by the c-*myc* oncogene. Like in the case of Ras immunoprecipitation, we also detected the 48-kD major band in the p53 immunoprecipiatets. Because the appearance of this 48-kD band was totally independent of p53 expression, this would be due to the nonspecific binding of 48-kD protein to the antibody.

DISCUSSION

The results presented reveal that (1) transforming Ras was more efficiently modificated at posttranslational level than normal one; (2) the c-*myc* oncogene inhibited the posttranslational modification of Ras and kept it as cytosolic precursor; and (3) either wild-type or mutant p53 did not affect the posttranslational modification of Ras, in spite of the fact that, like *Myc*, mutant p53 cooperates with Ras in transformation.

Mutation of the c-H-*ras* gene leading to the substitution of Val^{12} for Gly or Leu^{61} for Gln significantly increases the transforming activity of Ras. These mutations result in the decrease in GAP-mediated GTPase activity of Ras, thereby keep Ras active [Lowy and Willumsen, 1993; Weinberg, 1989]. Although we do not know how leucine substitution for Gln^{61} affects the posttranslational modification of Ras, our finding that one of the transforming Ras harboring valine substitution for Gly^{12} was more efficiently modificated and converted into membrane-bound forms than normal Ras may also account for the enhanced biological activities of transforming Ras. Since membrane localization of Ras is crucial for the membrane recruitment of Raf, that triggers MAP kinase cascade downstream of Ras [Stokoe et al., 1994], increase in the efficiency of membrane localization of transforming Ras may have synergistic effects with decreased GTPase activity on enhancing the biological activities of Ras.

The mechanism underlying the increase in the efficiency of posttranslational modification of transforming Ras is not clear. Purification of Ras farnesyltransferase demonstrates that the enzyme consists of two subunits, and that farne-

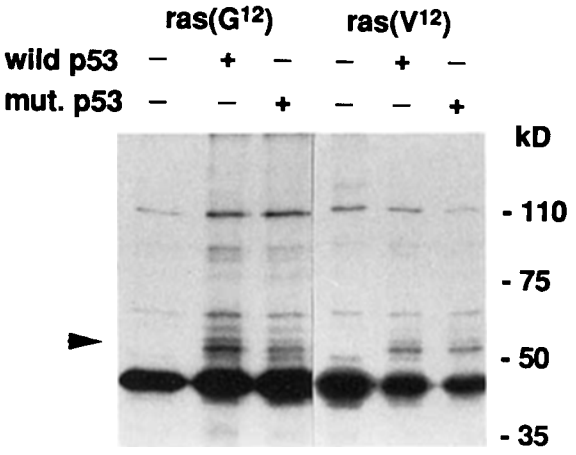

Fig. 5. Expression of wild-type p53 and mutant p53 in NIH3T3 cells harboring pMTcHr(G^{12}) or pMTcHr(V^{12}). Cells that had been transfected with pCDM8-p53W or pCDM8-p53M were cultured in a medium containing $CdCl_2$ for 24 h, labeled with [^{35}S]methionine/cysteine for 1 h; 1.5 mg of the total cell extracts was immunoprecipitated with anti-p53 monoclonal antibody, and the immune complexes were separated on a 10% SDS–polyacrylamide gel. *Arrowhead,* position of p53 proteins. Positions of the marker proteins were also indicated in kD. For further details, see Materials and Methods.

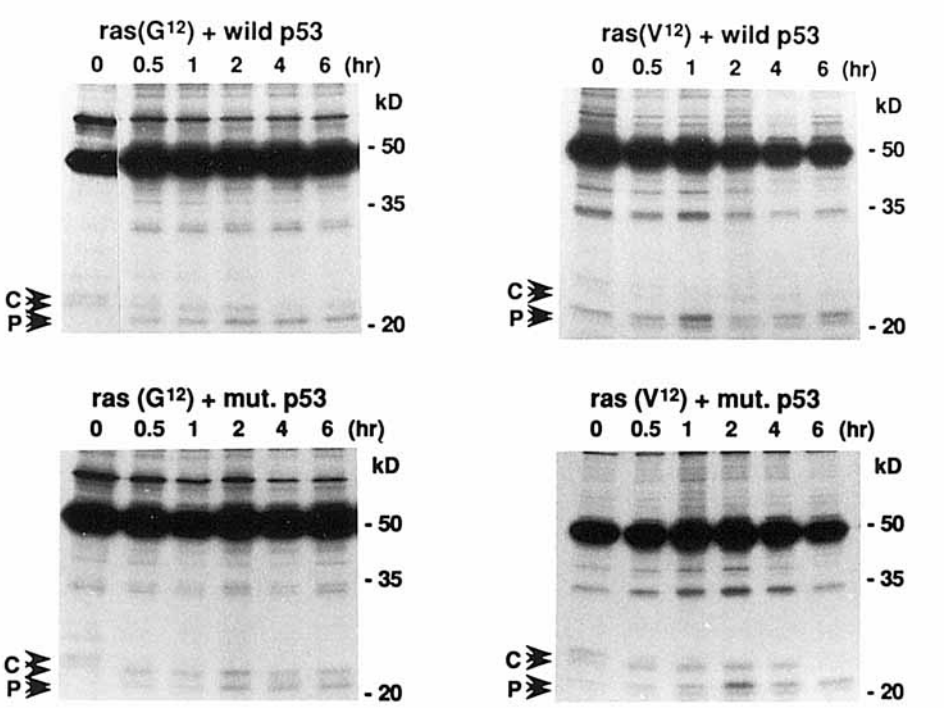

Fig. 6. Effects of p53 expression on the posttranslational modification of Ras in NIH3T3 cells harboring pMTcHr(G^{12}) or pMTcHr(V^{12}). Cells that had been transfected with pCDM8-p53W or pCDM8-p53M were cultured in a medium containing $CdCl_2$ for 24 h, labeled with [^{35}S]methionine/cysteine for 30 min, washed, and continued to culture for the indicated time in the presence of large excess of nonradiolabelled amino acids; 1.5 mg of the total cell extracts was immunoprecipitated with anti-Ras monoclonal antibody, and the immune complexes were separated on a 12.5% SDS–polyacrylamide gel. *Arrowheads P and C,* membrane-bound Ras and the cytoplasmic precursor of Ras, respectively. Positions of the marker proteins were also indicated in kD. For further details, see Materials and Methods.

syltransferase and geranylgeranyltransferase share a common α subunit whereas peptide-binding β-subunit is different between the two enzymes [Seabra et al., 1991]. Ras farnesyltransferase recognizes Cys-AAX motif where A stand for aliphatic amino acids and X can be any amino acid [Reiss et al., 1990]. The facts that both normal and transforming c-H-Ras proteins harbor the same amino acid sequence at C-terminus, Cys-Val-Leu-Ser, and that farnesyltransferase activity is completely inhibited by the Cys-Val-Leu-Ser tetrapeptide [Reiss et al., 1990], make it unlikely that normal and transforming Ras have different affinities to Ras farnesyltransferase. On the other hand, farnesylation of Ras is associated with the cleavage of C-terminal 3-amino acids and methylation of COOH group of the cysteine [Gutierrez et al., 1989]. Therefore, it would be of interest how valine substitution for Gly^{12} or leucine substitution for Gln^{61} affect the proteolytic cleavage of C-terminal 3-amino acids and methylation of newly produced C-terminal cysteine.

Recently, Jackson et al. [1994] reported that polylysine stretch of K-Ras 4B near C-terminus facilitated the membrane localization of protein, although the presence of Cys-AAX motif at C-terminus was sufficient for isoprenylation. As c-H-Ras does not contain polylysine stretch but undergoes palmitoylation that compensate for the polybasic domain of K-Ras4B [Hancock et al., 1989, 1990], one possibility is that different state of palmitoylation between normal and transforming c-H-Ras result in the different efficiency of membrane binding. In fact, farnesylation of Ras is essential for the membrane binding and transforming activity, but further palmitoylation is necessary for the tight association with plasma membrane [Cadwallader et al., 1994]. To explore this possibility, we have also labeled the cells with [^{14}C]palmitic acid and investigated the palmitoylation of Ras. However, incorporation of [^{14}C]palmitic acid into Ras was too low to distinguish the difference in palmitoylation between normal and transforming Ras.

The c-*myc* and the mutant p53 genes are known as oncogenes that cooperate with the c-H-*ras* oncogene in transformation [Land et al., 1983]. Interestingly, the c-*myc* oncogene was

found to suppress the posttranslational modification of Ras, whereas mutant p53 did not. The fact that the c-*myc* oncogene also induces programmed cell death in fibroblasts [Evan et al., 1992] may raise the possibility that impairment of the posttranslational modification of Ras by the c-*myc* oncogene is the consequence of the apoptotic activity of the c-*myc* oncogene. However, wild-type p53 also induces apoptosis in thymocytes [Clarke et al., 1993; Lowe et al., 1993], but it did not affect the posttranslational modification of Ras. Thus, inhibition of the posttranslational modification of Ras by the c-*myc* oncogene cannot simply be explained by the ability of inducing apoptosis, but by some other properties of the *myc* oncogene.

ACKNOWLEDGMENTS

We thank Dr. S. Nakamura and Dr. S. Hattori (National Institute of Neurosciences, Japan) for pMTcHr(G^{12}) and pMTcHr(V^{12}), Dr. A. Horwich (Yale University) for pFRSV, Dr. J. Cleveland (St. Jude Children's Research Hospital) for fpGV-c-*myc*, Dr. Y. Dobashi (Tokyo University) for pSV2-γ-actin, Dr. P. Berg (Stanford University) for pSV2*-hph, and Dr. K. Morishita (National Cancer Institute, Japan) for pCDM8-p53W, pCDM8-p53M, and psi3 cells.

REFERENCES

Buday L, Downward J (1993): Epidermal growth factor regulates $p21^{ras}$ through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. Cell 73:611–620.

Cadwallader KA, Paterson H, Macdonald SG, Hancock JF (1994): N-terminally myristoylated ras proteins require palmitoylation or a polybasic domain for plasma membrane localization. Mol Cell Biol 14:4722–4730.

Chardin P, Camonis JH, Gale NW, Aelst LV, Schlessinger J, Wigler MH, Bar-Sagi D (1993): Human Sos1: A guanine nucleotide exchange factor for ras that binds to Grb2. Science 260:1338–1343.

Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML, Wyllie AH (1993): Thymocyte apoptosis induced by p53-dependent and independent pathway. Nature 362:849–852.

Davis LG, Dibner MD, Battey JF (1986): Calcium phosphate transfection of nonadherent and adherent cells with purified plasmids. In "Basic Methods in Molecular Biology." New York: Elsevier Science, pp 286–289.

Dean M, Cleveland JL, Rapp UR, Ihle JN (1987): Role of myc in the abrogation of IL3 dependence of myeloid FDC-P1 cells. Oncogene Res 1:279–296.

Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, Weinberg RA (1993): Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature 363:45–51.

Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ, Hancock DC (1992): Induction of apoptosis in fibroblasts by c-myc protein. Cell 69:119–128.

Gale NW, Kaplan S, Lowenstein EJ, Schlessinger J, Bar-Sagi D (1993): Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras. Nature 363: 88–92.

Gutierrez J, Magee AI, Marshall CJ, Hancock JF (1989): Post-translational processing of $p21^{ras}$ is two-step and involves carboxy-methylation and carboxy-terminal proteolysis. EMBO J 8:1039–1098.

Hancock JF, Magee AI, Childs JE, Marshall CJ (1989): All ras proteins are polyisoprenylated but only some are palmitoylated. Cell 57:1167–1177.

Hancock JF, Paterson H, Marshall CJ (1990): A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize $p21^{ras}$ to the plasma membrane. Cell 63:133–139.

Hollstein M, Sidransky D, Vogelstein B, Harris CC (1991): p53 mutations in human cancers. Science 253:49–53.

Jackson JH, Li JW, Buss JE, Der CJ, Cochrane CG (1994): Polylysine domain of K-ras 4B protein is crucial for malignant transformation. Proc Natl Acad Sci USA 91:12730–12734.

Land H, Parada LF, Weinberg RA (1983): Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304:596–602.

Li N, Batzer A, Daly R, Yajnik V, Skolnik E, Chardin P, Bar-Sagi D, Margolis B, Schlessinger J (1993): Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signaling. Nature 363: 85–88.

Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T (1993): p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 362:847–849.

Lowy DR, Willumsen BM (1993): Function and regulation of ras. Annu Rev Biochem 62:851–891.

Lu X, Park SH, Thompson TC, Lane DP (1992): ras-induced hyperplasia occurs with mutation of p53, but activated ras and myc together can induce carcinoma without p53 mutation. Cell 70:153–161.

Moodie SA, Willumsen BM, Weber MJ, Wolfman A (1993): Complex of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. Science 260:1658–1661.

Oliver JP, Raabe T, Henkemeyer M, Dickson B, Mbamalu G, Margolis B, Schlessinger J, Hafen E, Pawson T (1993): A Drosophila SH2-SH3 adaptor protein implicated in coupling the sevenless tyrosine kinase to an activator of ras guanine nucleotide exchange, Sos. Cell 73:179–191.

Reiss Y, Goldstein JL, Seabra MC, Casey PJ, Brown MS (1990): Inhibition of purified $p21^{ras}$ farnesyl:protein transferase by cys-AAX tetrapeptides. Cell 62:81–88.

Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD, Downward J (1994): Phosphatidylinositol-3-OH kinase as a direct target of ras. Nature 370:527–532.

Roussel MF, Cleveland JL, Shurtleff SA, Sherr CJ (1991): Myc rescue of a mutant CSF-1 receptor impaired in mitogenic signaling. Nature 353:361–363.

Rozakis-Adcock M, Fernley R, Wade J, Pawson T, Bowtell D (1993): The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. Nature 363:83–85.

Ruley HE (1983): Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature 304:602–606.

Sambrook J, Fritsch EF, Maniatis T (1989): "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Seabra MC, Reiss Y, Casey PJ, Brown MS, Goldstein JL (1991): Protein farnesyltransferase and geranylgeranyltransferase share a common α subunit. Cell 65:429–434.

Shih TY, Weeks MO, Gruss P, Dhar R, Oroszlan S, Scolnick EM (1982): Identification of a precursor in the biosynthesis of the p21 transforming protein of Harvey murine sarcoma virus. J Virol 42:253–261.

Simon MA, Dodson GS, Rubin GM (1993): An SH3-SH2-SH3 protein is required for $p21^{Ras1}$ activation and binds to sevenless and Sos proteins in vitro. Cell 73:169–177.

Simonsen CC, Levinson AD (1983): Isolation and expression of an altered mouse dihydrofolate reductase cDNA. Proc Natl Acad Sci USA 80:2495–2499.

Sinn E, Muller W, Pattengale P, Tepler I, Wallace R, Leder P (1987): Coexpression of MMTV/v-Ha-ras and MMTV/c-*myc* genes in transgenic mice: Synergistic action of oncogenes in vivo. Cell 49:465–475.

Stokoe D, Macdonald SG, Cadwallader K, Symons M, Hancock JF (1994): Activation of raf as a result of recruitment to the plasma membrane. Science 264:1463–1467.

Vojtek AB, Hollenberg SM, Cooper JA (1993): Mammalian ras interacts directly with the serine/threonine kinase raf. Cell 74:205–214.

Warne PH, Viciana PR, Downward J (1993): Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. Nature 364:352–355.

Weinberg RA (ed) (1989): "Oncogenes and the Molecular Origin of Cancer." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Willumusen BM, Norris K, Papageorge AG, Hubbert NL, Lowy DR (1984): Harvey murine sarcoma virus p21 ras protein: Biological and biochemical significance of the cysteine nearest carboxy terminus. EMBO J 3:2581–2585.

Zhang X-F, Settleman J, Kyriakis JM, Takeuchi-Suzuki E, Elledge SJ, Marshall MS, Bruder JT, Rapp UR, Avruch J (1993): Normal and oncogenic $p21^{ras}$ proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature 364: 308–313.