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Posttranslational modification of the Ha-*ras* oncogene protein: Evidence for a third class of protein carboxyl methyltransferases

(protein methylation/transforming proteins/membrane attachment)

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ABSTRACT **The *ras* oncogene products require membrane localization for their function, and this is thought to be accomplished by the addition of a palmitoyl group to a cysteine residue near the carboxyl terminus of the nascent chain. A lipidated carboxyl-terminal cysteine residue is also found in sequence-related yeast sex factors, and in at least two cases, the α-carboxyl group is also methyl esterified. To determine if ras proteins are themselves modified by a similar type of methylation reaction, we incubated rat embryo fibroblasts transformed with p53 and activated Ha-*ras* oncogenes with L-[*methyl*-^{3}H]methionine under conditions in which the isotope was converted to the methyl donor *S*-adenosyl-L-[*methyl*-^{3}H]methionine. By using an assay that detects methyl ester linkages, we found that immunoprecipitated ras proteins are in fact esterified and that the stability of these esters is consistent with a carboxyl-terminal localization. This methylation reaction may be important in regulating the interaction of *ras* proteins with plasma membrane components. The presence of analogous carboxyl-terminal tetrapeptide sequences in other proteins may provide a general recognition sequence for lipidation and methylation modification reactions.**

Two types of protein carboxyl methyltransferases, enzymes that catalyze the transfer of the methyl group from *S*-adenosylmethionine to protein carboxylic acid groups, have been described. The first type (protein-glutamate methyltransferase, *S*-adenosyl-L-methionine:protein-L-glutamate *O*-methyltransferase, EC 2.1.1.24) has only been found in chemotactic bacteria, and it specifically and stoichiometrically methylates several glutamic residues on membrane-bound receptor proteins (1). The second class of methyltransferases (protein-D-aspartate methyltransferase, *S*-adenosyl-L-methionine:protein-D-aspartate *O*-methyltransferase, EC 2.1.1.77) is widely distributed in nature and substoichiometrically methylates a large variety of proteins and peptides (2). This latter enzyme is apparently specific for aspartic residues that have been covalently altered by isomerization and racemization reactions, and the products appear to be D-aspartate β-methyl esters and L-isoaspartate α-methyl esters (3, 4). The first class of methyltransferases functions to regulate the output of bacterial chemoreceptor proteins (5); the second class of enzymes appears to play a role in the metabolism of damaged proteins (6).

The possible existence of a third class of protein carboxyl methyltransferases is suggested by the structures of peptidyl mating factors from the jelly fungi *Tremella mesenterica* (tremerogen A-10) (7) and *Tremella brasiliensis* (tremerogen A-9291-I) (8). In both peptides, a carboxyl-terminal cysteine residue is present, which is modified by the addition of an oxidized farnesyl residue in a thioether linkage to the side-chain sulfur and by the addition of a methyl ester at the α-carboxyl group. The presence of this methyl ester could be accounted for by a distinct type of methyltransferase, perhaps specific for a lipidated terminal α-carboxyl group. The function of the methyl ester has not been established, but it has been shown that an unmethylated form of the tremerogen from *T. brasiliensis* is 200 times less active than the methylated peptide (8).

Preliminary evidence indicates that the **a** mating factor from the yeast *Saccharomyces cerevisiae* also contains a lipidated carboxyl-terminal cysteine residue (9). The possible presence of a methyl ester has not been determined, but the α-carboxyl group is blocked in a manner consistent with methylation at this site (9). The mRNA derived from both of the yeast genes for the **a** mating factor codes for three additional amino acids at the carboxyl terminus of the polypeptide (10). This result suggests that proteolytic processing occurs before the lipidation and the potential methylation reaction.

We were intrigued by recent reports that the structure of the carboxyl-terminal region of the mammalian *ras* oncogene products, as well as its counterpart in yeast, is similar to that of the yeast sex factors. Although the function of the intracellular GTP-binding *ras*-encoded proteins in cellular proliferation is distinct from that of the peptidyl mating factors, the mRNAs coding for the *ras* proteins specify a cysteine-containing carboxyl-terminal sequence of four amino acids that is similar to the sequence coded by the **a** mating factor gene (Table 1) (11). The cysteine residue in the *ras* protein appears to be lipidated by a palmitoyl group (12, 13), and this residue is crucial to the membrane localization and transforming activity of this protein (14). Additionally, there are yeast mutants that are defective in both *ras* and **a** mating factor function, and it appears that the deficiency in these strains may be related to a common pathway of posttranslational processing at the carboxyl terminus (15).

In this study we asked whether mammalian *ras* proteins are modified by a methylation reaction that may parallel the methylation of the *Tremella* sex factors. We provide evidence that activated Ha-*ras*-encoded proteins are in fact methylated and that both the degree of methylation and the stability of the methyl ester are consistent with a modification of the carboxyl-terminal α-carboxyl group.

METHODS AND MATERIALS

Cells. Primary rat embryo fibroblasts and a transformed fibroblast cell line (KH215 T22-4) prepared by cotransfection with plasmids containing activated human Ha-*ras* DNA (Gly-12 to Val-12 substitution, ref. 16) and murine p53 cDNA containing a linker-insertion mutation at amino acid position 215 (17) were a kind gift of Cathy Finlay, Philip Hinds, and

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Table 1. Comparison of carboxyl-terminal amino acid sequences of yeast mating factors, *ras* proteins, and other proteins

Protein	Carboxyl-terminal sequence
Yeast mating factors	
S. cerevisiae (**a** factor)	-Asp-Pro-Ala-Cys*
	-Asp-Pro-Ala-Cys-Val-Ile-Ala
T. brasiliensis (A-9291-I)	-Ser-Gly-Gly-Cys
T. mesenterica (A-10)	-Asn-Gly-Tyr-Cys
Ras proteins¶	
Human and rat Ha-*ras*-1	-Ser-Cys-Lys-Cys-Val-Leu-Ser
Chicken Ha-*ras*-1	-Asn-Cys-Lys-Cys-Val-Ile-Ser
Human K-*ras*-2A	-Ile-Lys-Lys-Cys-Ile-Ile-Met
Mouse K-*ras*-2A	-Ile-Lys-Lys-Cys-Val-Ile-Met
Human K-*ras*-2B	-Lys-Thr-Lys-Cys-Val-Ile-Met
Mouse K-*ras*-2B	-Arg-Thr-Arg-Cys-Thr-Val-Met
Human N-*ras*	-Gly-Leu-Pro-Cys-Val-Val-Met
Mouse N-*ras*	-Gly-Ser-Pro-Cys-Val-Leu-Met
Drosophila Dras1	-Arg-Phe-Lys-Cys-Lys-Met-Leu
Drosophila Dras2/64B	-Lys-Arg-Lys-Cys-Cys-Leu-Met
Dictyostelium Dras	-Lys-Lys-Gln-Cys-Leu-Ile-Leu
S. cerevisiae RAS1	-Gly-Gly-Cys-Cys-Ile-Ile-Cys
S. cerevisiae RAS2	-Gly-Gly-Cys-Cys-Ile-Ile-Ser
S. pombe SPRAS	-Cys-Val-Ile-Cys
Other proteins‖	
Bovine transducin (γ subunit)	-Lys-Gly-Gly-Cys-Val-Ile-Ser
Xenopus laevis nuclear lamin L_I	-Asn-Lys-Asn-Cys-Ala-Ile-Met
Human nuclear lamin A	-Pro-Gln-Asn-Cys-Ser-Ile-Met
Bovine retina cGMP phosphodiesterase (α subunit)	-Ser-Lys-Ser-Cys-Cys-Val-Gln
Rat brain G_o (α subunit)	-Leu-Arg-Gly-Cys-Gly-Leu-Tyr
Rat brain G_i (α subunit)	-Leu-Lys-Asp-Cys-Gly-Leu-Phe

G_o, guanine nucleotide-binding protein of unknown function; G_i, guanine nucleotide-binding protein that inhibits adenylate cyclase.
*The carboxyl-terminal cysteine residue appears to be modified by a S-alkylation reaction and does not appear to contain a free terminal carboxyl group (9).
†Sequence deduced from the **a** factor genes (10). This sequence would not reflect any posttranslational modifications.
‡The carboxyl group of the cysteine residue is linked in a methyl ester, and the sulfhydryl group is linked in a thioether to a hydroxylated farnesyl group (8).
§The carboxyl group of the cysteine residue is methyl esterified; the side chain is modified by a hydroxylated farnesyl group (7).
¶Sequences are from DNA sequences and do not reflect posttranslational modifications (11).
‖Sequences are derived from cDNA sequences and do not reflect posttranslational modifications; see text for references.

Arnold Levine (Princeton University) and were prepared as previously described (18). Cells were grown to 90% confluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum on 100-mm plates incubated at 37°C in humidified air containing 5% CO_2.

Incubation with [*methyl*-^{3}H]Methionine and [^{35}S]Methionine. The growth medium was aspirated from one 100-mm Petri dish of cells, and adherent fibroblasts were washed with 5 ml of phosphate-buffered saline (PBS). After removal of the PBS, 5.0 ml of labeling medium was added. This amount of medium was prepared by removing the ethanol/water solvent from 1 mCi (1 Ci = 37 GBq) of L-[*methyl*-^{3}H]methionine (80 Ci/mmol, New England Nuclear) or a similar preparation of isotope in 50 mM *N*-tris(hydroxymethyl)methylglycine, pH 7.4/10 mM 2-mercaptoethanol in a Savant Speedvac apparatus. The residue was dissolved in methionine-free Dulbecco's modified Eagle's medium/2% fetal bovine serum. Cells were incubated at 37°C in a 5% CO_2 incubator for 3 hr. The labeled medium was then removed by aspiration, and the cells were washed with 5 ml of PBS at 0°C. Cells were removed from the dish with a rubber policeman into 10 ml of cold PBS and were collected by centrifugation at 2000 rpm (900 × *g*) for 5 min. After the removal of the supernatant, the cell pellets (≈0.1 ml for the transformed cell lines and 0.04 ml for the primary fibroblasts) were frozen at −70°C.

Cells were also labeled with L-[^{35}S]methionine [1097 Ci/mmol (ICN), containing ≈15% [^{35}S]cysteine] by using a similar protocol in which 500 μCi of label was present in the 5 ml of medium.

Preparation of Cell Extracts. Packed cell pellets were resuspended in 1.0 ml of lysis buffer [10 mM Tris·HCl, 150 mM NaCl, 1% (wt/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) sodium dodecyl sulfate, 1 μM pepstatin, 100 units of aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride (pH 7.2)] at 0°C. Cells were disrupted by 10–15 passages through a 3.8-cm 21-gauge stainless steel needle. Unbroken cells and large debris were removed by centrifugation at 1500 rpm (500 × *g*) for 15 min, and the supernatant was then centrifuged at 8000 rpm (11,000 × *g*) for 15 min.

Immunoprecipitation. Cell extracts (350 μl) were mixed with 1 μg of HPLC-purified IgG fraction of Y13-259 rat monoclonal antibodies directed against v-Ha-*ras* (Oncogene Science, Manhasset, NY; ref. 19) and 65 μl of a 50% (vol/vol) suspension of staphylococcal protein A-Sepharose beads coated with a rabbit antibody to rat IgG. These coated beads were prepared by incubating 1 ml of a 50% (vol/vol) suspension of protein A-Sepharose CL-4B beads (Sigma; washed and suspended in lysis buffer; nominal binding capacity of 20 mg of human IgG per ml) with 0.25 ml of a rabbit antiserum to rat IgG (43.9 mg/ml of total protein; 4.0 mg/ml of antibody protein; heavy and light chain specific; Cappel/Cooper Biomedical, West Chester, PA) for 6 hr at 22°C, washing twice in lysis buffer and resuspending to 50% (vol/vol), and finally adding an equal volume of 50% (vol/vol) uncoated protein A-Sepharose beads. After incubation with or without antibody at 4°C overnight with constant agitation, the beads were recovered by centrifugation and washed five times with 0.75 ml of lysis buffer at 0°C.

Sodium Dodecyl Sulfate Gel Electrophoresis. Polyacrylamide slab gels (separating gel: 12% acrylamide, 15-cm wide, 8-cm high, 0.14-cm thick; stacking gel: 5% acrylamide, containing 13 wells 0.6-cm wide and 1.85-cm deep) were made using the buffer system described by Laemmli (20). The sample buffer was 1% sodium dodecyl sulfate/280 mM 2-mercaptoethanol/62 mM Tris Cl/5% glycerol/0.001% bromphenol blue, pH 6.8. Electrophoresis was performed for 3 hr at a constant current of 35 mA. Gels were stained for 1 hr with Coomassie blue dye dissolved in 25% isopropanol (vol/vol) and 10% acetic acid (wt/vol). After they were destained overnight in 10% acetic acid (wt/vol), gels containing tritium label were photographed and dried under reduced pressure at 60°C. Destained gels containing ^{35}S label were rinsed in 5% methanol, soaked for 30 min in 1 M sodium salicylate, rinsed with water, dried, and analyzed by fluorography.

Vapor-Phase Equilibration Assay for Methyl Esters. This assay is based on the transfer of volatile methanol from base-hydrolyzed protein methyl esters to scintillation fluid (21). Dried gel lanes were cut into 0.2-cm or 0.3-cm slices and were mixed with 50 μl of 1 M sodium hydroxide in a 1.5-ml polyethylene microcentrifuge tube. This tube was carefully lowered into a 6-ml plastic scintillation vial containing 2.4 ml of aqueous counting fluid (Liquiscint; National Diagnostics,

Somerville, NJ). After 24 hr at 37°C, radioactivity was assayed by liquid scintillation spectrometry. The efficiency of this assay was determined in a control experiment in which 10 μl of [^{14}C]methanol was added to 50 μl of 1 M sodium hydroxide in the protocol described above. Approximately 83% of the radioactivity present in the original sample was recovered after vapor-phase transfer to the scintillation cocktail under these conditions.

Determination of Total Radioactivity in Gel Slices. Dried gel sections (0.3 cm) were hydrated with 0.1 ml of water and mixed with 1 ml of 30% (vol/vol) hydrogen peroxide. After they were heated overnight at 68°C to solubilize the gel, 10 ml of scintillation fluid (Liquiscint; National Diagnostics) was added, and the radioactivity was determined by liquid scintillation spectrometry.

RESULTS

Expression of *Ras* Genes in p53/*ras* Oncogene-Transfected Rat Embryo Fibroblasts. Rat embryo fibroblasts can be transformed by cotransfection with the p53 nuclear oncogene and the activated Ha-*ras* oncogene (18). Immunoprecipitation of extracts of [^{35}S]methionine-labeled fibroblasts indicated that these cells contain significant levels of *ras* oncogene proteins (Fig. 1). In contrast, experiments with primary rat embryo fibroblasts showed that the endogenous *ras* proteins were present at a level estimated from densitometry of the autoradiograph to be only about 0.2% of that of the transformed cells.

Occurrence of a Stable Class of Carboxyl-Methylated Proteins in Fibroblasts. Rat primary embryo fibroblasts and the transformed line described above were incubated in the presence of methionine labeled with ^{3}H in the methyl group. Radioactivity would be expected to be incorporated into proteins both as methionyl residues and as methyl groups that modify the side chains of various amino acids (22). The latter reaction is dependent upon the conversion of methionine to the methyl group donor *S*-adenosylmethionine, a reaction catalyzed by a synthetase present in human and rat fibroblasts (23).

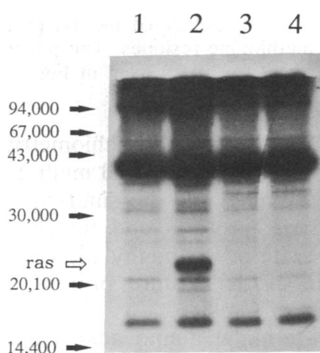

FIG. 1. Expression of the *ras* oncogene product in primary rat embryo fibroblasts and a Ha-*ras*/p53 oncogene-transformed cell line (KH215 T22-4). Extracts of [^{35}S]methionine-labeled cells were immunoprecipitated with the Y13-259 antibody. The final pellet (≈50 μl) was added to 18 μl of a 4-fold concentrated sodium dodecyl sulfate sample buffer and was heated for 5 min at 90°C; 20-μl aliquots were electrophoresed. After staining and salicylate treatment, the dried gel was analyzed by fluorography for 18 hr at −70°C with x-ray film. Lanes: 1 and 2, extracts of Ha-*ras*/p53 transformed cells; 3 and 4, primary rat embryo fibroblasts. Lanes 1 and 3 show experiments done in the absence of anti-ras antibody. The positions of polypeptide molecular weight markers (Pharmacia; including phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin) electrophoresed in parallel lanes are indicated with arrows, as is the position of the *ras* polypeptide at an approximate polypeptide M_r of 23,200.

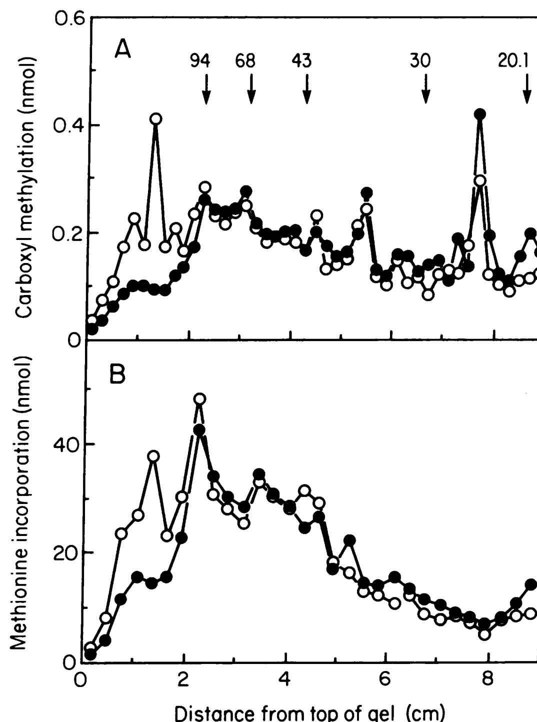

FIG. 2. Methyl ester and methionine incorporation in transformed and control rat embryo fibroblasts incubated with [*methyl*-^{3}H]methionine. Extracts from a radiolabeled primary culture of rat embryo fibroblasts and from a *ras*/p53 transformed line (KH215 T22-4) were prepared and mixed with sodium dodecyl sulfate sample buffer as described in *Methods and Materials* and the legend to Fig. 1. Approximately 19 μg of protein from the control fibroblasts and 41 μg from the transformed fibroblasts was applied to a 12% polyacrylamide gel. (*A*) Coomassie-stained dried lanes were cut into 2-mm slices and assayed for methyl esters by using a vapor-phase equilibration assay. Carboxyl methyl esters were quantitated for control fibroblast polypeptides (○) and transformed fibroblast polypeptides (●) as nmol of [^{3}H]methyl groups per gel slice per mg of protein. Specific radioactivity was determined by measuring the specific radioactivity of methionine after fractionation of *o*-phthalaldehyde-derivatized amino acids from acid hydrolysates of the total protein fraction. The values for control and transformed fibroblasts were 36.1 cpm/pmol and 191.6 cpm/pmol, respectively. The positions of molecular weight standards (in $M_r \times 10^{-3}$) used in Fig. 1 are indicated by arrows. (*B*) Methionine incorporation was determined from the total radioactivity assayed in 3-mm slices. Quantitation in terms of nmol incorporated per gel slice per mg of protein was done as described above.

Radiolabeled proteins from crude lysates of these fibroblasts were fractionated by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate (Fig. 2). We chose to use the electrophoresis buffer system developed by Laemmli (20) because the separation gel has an alkaline pH (8.5), which results in the hydrolysis of protein methyl esters that demethylate by way of succinimide mechanisms (such as D-aspartate β-methyl esters and L-isoaspartate α-methyl esters) but not of those of more stable esters such as L-glutamate γ-methyl esters or esters of carboxyl-terminal amino acids (2, 24). In this way, we have selected against detecting methyl esters of altered aspartate residues, which have been found on a large variety of proteins in all cell types examined (2). To distinguish radioactivity present in the class of stable protein methyl esters from that present in methionine residues or as base-stable methyl groups on the side chains of arginine, histidine, or lysine residues, a vapor-phase equilibration assay that measures volatile radioactivity produced as [^{3}H]methanol from base-hydrolyzed ester linkages was performed.

As shown in Fig. 2A, volatile radioactivity was detected in both control and transformed fibroblasts at various polypeptide molecular weights, including a major peak at an approximate M_r of 23,000. The total radioactivity incorporated into the polypeptides of these cells is shown in Fig. 2B. A comparison of the total and volatile radioactivity suggests that less than 1% of the total radioactivity from the [^{3}H]methionine labeling was present in a form made volatile by base treatment. Methionine incorporation and carboxyl methylation were approximately the same in the control and transformed cells. It is significant to note that the proportion of volatile radioactivity was greatest in the M_r 23,000 region, and it appears that polypeptides of this size may be major sites for stable methyl esterification reactions in the cell. Although the molecular weight of the methylated polypeptides in the M_r 23,000 region in the control cell line is similar to that of the mammalian *ras* proteins, it does not appear that a major part of this radioactivity is present in *ras* proteins since the increased incorporation in transformed cells is not proportional to the increased levels of *ras* protein (Fig. 1). Similar results have been obtained using baby rat kidney fibroblasts transformed with either activated or unactivated *ras*.

Immunoprecipitation of *ras* Proteins from [*methyl*-^{3}H]Methionine-Labeled Cells. When *ras* proteins were immunoprecipitated from cell lysates of the transformed fibroblasts labeled with [*methyl*-^{3}H]methionine and fractionated by sodium dodecyl sulfate gel electrophoresis, radioactivity was detected in a polypeptide corresponding to *ras* with an approximate M_r of 23,000 (Fig. 3B). When gel slices were analyzed for methyl esters by the vapor-phase assay, a peak of radioactivity was detected that comigrated with the peak of total radioactivity (Fig. 3A). No peak of radiolabeled polypeptide was detected at the position of the *ras* protein in control experiments where the monoclonal antibody to *ras* was not present in the immunoprecipitation reaction, and no peak was present in the immunoprecipitate of control untransformed fibroblasts.

An estimate of the stoichiometry of the methylation of *ras* proteins was made by comparing the total radioactivity (incorporation into both methionine and methyl esters) and the base-labile, volatile radioactivity (incorporation only into methyl esters) in the immunoprecipitated *ras* protein. The DNA sequence of the human Ha-*ras*-1 gene codes for five methionine residues, including the initiator methionine (11). If the initiator methionyl residue is cleaved, the stoichiometric incorporation of one methyl ester per polypeptide chain would be expected to result in a ratio of base-labile, volatile radioactivity to total radioactivity of 1:5. The results of the experiment shown in Fig. 3 indicate that the ratio is very close to 1:7. If the rates of turnover of *ras* protein and *ras* methyl ester are the same, this ratio indicates a stoichiometry of 0.67 methyl group per *ras* monomer. Preliminary labeling experiments in the presence of puromycin indicate that methylation of *ras* can occur in the absence of protein synthesis, and the actual stoichiometry may be lower if protein turnover is slow compared to the loss of methyl esters.

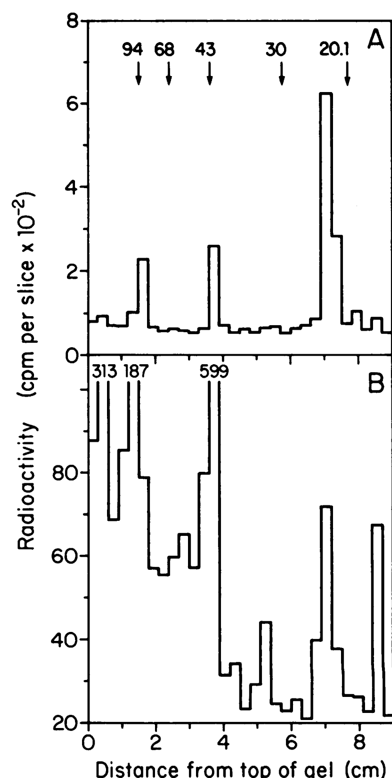

FIG. 3. Immunoprecipitation of Ha-*ras* proteins from transformed fibroblasts labeled with [*methyl*-^{3}H]methionine. Immunoprecipitates were denatured in sodium dodecyl sulfate, and the polypeptides were separated as shown in Fig. 1. Dried gel slices (3 mm) were assayed for base-labile volatile radioactivity (*A*) and for total radioactivity (*B*). The amount of methyl groups incorporated into the *ras* polypeptide was calculated from the radioactivity migrating from 6.9 to 7.5 cm from the top of the gel. By assuming a background of 40 cpm and 83% efficiency in methanol transfer from the gel slice to the scintillation fluid, the *ras* polypeptide band contains 1000 cpm as methyl esters. The total radioactivity (which includes both methyl esters and methionine residues in the protein) in these same fractions is 6987 if a background of 2000 cpm is assumed. These results suggest that approximately one radiolabeled methyl group is present for every six labeled methionine residues. The positions of molecular weight standards (in $M_r \times 10^{-3}$) used in Fig. 1 are indicated by arrows.

DISCUSSION

We show here that the activated Ha-*ras* oncogene product is methyl esterified in the transformed rat embryo fibroblasts in a reaction that appears to represent the activity of a third class of eukaryotic protein methyltransferases. The only class of eukaryotic carboxyl methyltransferases previously characterized recognizes L-isoaspartic and D-aspartic residues. The methylated *ras* oncogene polypeptide does not appear to be a product of this enzyme because aspartate methyl esters are generally hydrolyzed during sodium dodecyl sulfate gel electrophoresis at alkaline pH values, and their incorporation is markedly substoichiometric (2). Based on the presence of a carboxyl-terminal methyl ester in the sex factors of the jelly fungi and the similarity of the carboxyl termini of both *ras* proteins and fungal sex factors, we propose that the site of *ras* methylation is at the carboxyl-terminal residue of the mature protein, possibly at the α-carboxyl group of Cys-186.

The carboxyl-terminal amino acid sequences predicted from the cDNA for various *ras* proteins show little similarity except for the terminal four amino acids, where an invariant cysteine residue is followed by two generally aliphatic residues and a terminal cysteine, serine, leucine, or methionine residue (Table 1). The invariant cysteine residue is important for activity and is thought to be the site of palmitoylation by way of a thioester linkage (12–14). A similar sequence is predicted for the *S. cerevisiae* **a** mating factor (10), where the cysteine residue also appears to be lipidated (9), as well as the γ subunit of transducin from bovine retina (25) and the lamin type B protein from the nuclear membrane of *Xenopus* (26). Direct chemical analysis of purified **a** factor indicates that the last three amino acids are removed to expose the cysteine residue at the carboxyl terminus (9). It is not yet clear if the terminal three amino

acids are also cleaved in the *ras* proteins, transducin, and lamin B. The possibility of a general proteolytic processing step is suggested, however, by the homology of the sequence in this region. It is possible that all of these proteins share a similar structure at the carboxyl terminus—the presence of a tetrapeptide sequence that can be modified by cleavage, lipidation, and methylation.

There are other proteins with deduced primary sequences that include a cysteine residue in the fourth position from the carboxyl terminus (Table 1). For example, cDNAs of the α subunits of G_o and G_i (but not G_s) [where G_o, G_i, and G_s are guanine nucleotide-binding proteins of unknown function (G_o) or that inhibit (G_i) or stimulate (G_s) adenylate cyclase] from rat brain encode a cysteine residue three residues from the carboxyl terminus (27), and similar sequences are predicted for the related human (28) and bovine (29) species. A similar sequence has been deduced for the cGMP phosphodiesterase from bovine retina (30) and for the human lamin A protein (32). In addition, several proteins contain carboxyl-terminal cysteine residues that are targets of lipidation reactions. These include the rat, mouse, and yeast *ras*-related YPT1 proteins (31, 33).

We were very interested to note that evidence exists in the literature for the methyl esterification of two of the non-*ras* proteins listed in Table 1 that contain the carboxyl-terminal Cys-Xaa-Xaa-Xaa "fingerprint." In the first case, the α subunit of the cGMP phosphodiesterase appears to be methylated at a carboxyl group in bovine rod outer segments (34). Second, evidence has been presented that the nuclear lamin B protein from mouse tissues is also methyl esterified (35). Although the sites of esterification of these molecules are not known, it is tempting to speculate that these proteins are methylated in a similar fashion as the Ha-*ras*-encoded protein described here. We can propose that this type of carboxyl-terminal sequence may be a signal for the cell to initiate the methylation, lipidation, and cleavage reactions that may result in the targeting of these proteins for membrane attachment. For example, we note that there is no apparent hydrophobic region in the deduced amino acid sequence for the nuclear lamin protein (26), and a posttranslational lipidation/methylation reaction may account for the association of this protein with the nuclear membrane (36, 37).

What can we say at this point on the physiological function of a carboxyl-terminal methylation reaction? The conversion of a carboxylate anion at the carboxyl terminus to a methyl ester would certainly increase the hydrophobicity of this residue and may provide an additional hydrophobic surface for membrane attachment. It is also possible that the chemical reactivity of the carboxylate anion is eliminated by the methylation reaction. Finally, turnover of the palmitoyl group has been found in the N-*ras* protein in human cells (38), and a similar turnover of the methyl group may have a regulatory function in these proteins. Thus, the dynamic reversible methylation of the *ras* proteins could play an important role itself in the modulation of their signaling properties.

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