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Prenylation of Mammalian Ras Protein in *Xenopus* Oocytes

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Ras protein requires an intermediate of the cholesterol biosynthetic pathway for posttranslational modification and membrane anchorage. This step is necessary for biological activity. Maturation of *Xenopus laevis* oocytes induced by an oncogenic human Ras protein can be inhibited by lovastatin or compactin, inhibitors of the synthesis of mevalonate, an intermediate of cholesterol biosynthesis. This inhibition can be overcome by mevalonic acid or farnesyl diphosphate, a cholesterol biosynthetic intermediate downstream of mevalonate, but not by squalene, an intermediate after farnesyl pyrophosphate in the pathway. This study supports the idea that in *Xenopus* oocytes, the Ras protein is modified by a farnesyl moiety or its derivative. Furthermore, an octapeptide with the sequence similar to the C-terminus of the c-H-*ras* protein inhibits the biological activity of Ras proteins in vivo, suggesting that it competes for the enzyme or enzymes responsible for transferring the isoprenoid moiety (prenylation) in the oocytes. This inhibition of Ras prenylation by the peptide was also observed in vitro, using both *Saccharomyces cerevisiae* and *Xenopus* oocyte extracts. These observations show that *Xenopus* oocytes provide a convenient in vivo system for studies of inhibitors of the posttranslational modification of the Ras protein, especially for inhibitors such as peptides that do not penetrate cell membranes.

Three Ras proteins, N-, H-, and K-p21^{ras}, are members of a family of low-molecular-weight, guanine nucleotide-binding proteins that have the ability to transform cells when the proteins are mutated at one of several key residues. To be active, Ras must associate with the inner surface of the plasma membrane. There is a strong similarity between the posttranslational modification of the carboxyl terminus of *Saccharomyces cerevisiae* a-factor (1) and those of Ras proteins. The last four amino acids of both proteins conform to the consensus sequence of C-A-A-X, where C is Cys, A is commonly an aliphatic amino acid, and X is any amino acid. Recently, it has been shown that a series of posttranslational modifications is required for both proteins: (i) attachment of an isoprenoid-derived lipid to Cys-186 via a thioether linkage (5, 8, 13); (ii) cleavage of the three C-terminal amino acids (A-A-X), leaving Cys at the carboxyl terminus (6); and (iii) carboxymethylation of this Cys (6). Although palmitoylation was thought to take place at the cysteine residue in C-A-A-X and to contribute to membrane attachment of Ras proteins (4, 14), it is now known that palmitoylation occurs at nearby Cys residues and enhances membrane association only after prenylation of the C-terminal cysteine (9). Prenylation is critical for membrane association of Ras proteins, and this modification, in turn, is necessary for oncogenic Ras proteins to exhibit transformation activity.

We had shown previously that compactin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase (an enzyme in the cholesterol biosynthetic pathway), was able to inhibit the biological activity of an oncogenic protein, c-H-*ras*$^{Val-12}$ protein, in *Xenopus laevis* oocytes (13). This inhibition could be reversed by the addition of mevalonic acid (the product of the reaction catalyzed by HMG CoA reductase). This result established that mevalonic acid or, more likely, a polyisoprenoid derived from mevalonate was required for the biological activity of Ras protein. This interpretation was supported by direct demonstration of the prenylation of mammalian Ras protein (9) and the identification of the prenyl moiety as farnesyl (5). In this study, we examined the ability of downstream intermediates to rescue the effect of inhibition of mevalonate synthesis (Fig. 1). These results established that farnesyl diphosphate (FPP) (or its derivative) is the substrate for the enzyme that transfers the prenyl moiety to the Ras protein in vivo in the oocytes. In addition, a peptide containing the consensus sequence of the carboxyl terminus of the Ras proteins was able to inhibit the transforming activity in vivo and farnesylation in vitro of c-H-*ras*$^{Val-12}$ protein, suggesting the potential of peptide-based molecules as inhibitors of protein prenylation.

MATERIALS AND METHODS

Materials. Lovastatin (Mevacor) was a generous gift from A. Alberts, Merck, Sharp & Dohme, West Point, Pa. Squalene was obtained from Sigma, St. Louis, Mo. Pentapeptide KCVLS was provided by Chi-Ching Yang of Protos, Emeryville, Calif. The octapeptides were obtained from Multiple Peptide Systems, San Diego, Calif. Isopentenyl diphosphate (IPP) was obtained from Amersham Life Science Products. FPP was a gift from Dale Poulter, University of Utah, Salt Lake City, Utah.

Protein expression and purification. A synthetic gene coding for mammalian c-H-*ras*$^{Val-12}$ protein was expressed using a *trp* promoter in *Escherichia coli* HB101 and purified as described previously (11). The protein was maintained in buffer A (50 mM β-glycerophosphate, 3 mM $MgCl_2$ [pH 7.5]) before injection into *Xenopus* oocytes. Protein concentrations were determined by the Bradford assay (3), using bovine serum albumin as a standard.

Microinjection. *X. laevis* oocytes were microinjected by Mike Wu and Associates, Berkeley, Calif. Stage VI oocytes were removed from the animals and injected with 12.5 or 25 nl of sample into the cytoplasm. After incubation at 21°C in Ringer solution, germinal vesical breakdown (GVBD) was scored by noting the appearance of a white spot in the animal hemisphere. Mevalonate depletion was achieved by injecting the oocytes with 25 nl of 2 mM lovastatin and incubating for 1 h at room temperature. In some experiments, a mixture of

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TABLE 1. Reactivation of human c-H-*ras*$^{Val-12}$ protein in mevalonate-deprived *Xenopus* oocytes[a]

Expt no.	Amt (nl) of 2 mM lovastatin	Amt (nl) of 0.2 mM c-H-*ras*$^{Val-12}$	Amt (nl) of 35 mM FPP or IPP	No. of oocytes showing GVBD/total no. of treated oocytes (incubation time)
1	25	0	0 FPP	0/6
2	0	12.5	0 FPP	4/4 (14 h)
3	25	12.5	0 FPP	0/6
4	25	12.5	12.5 IPP (41 mM)	6/6
5	25	12.5	12.5 IPP (82 mM)	6/6
6	0	12.5	12.5 IPP (82 mM)	5/6
7	0	0	12.5 FPP	0/6
8	0	12.5	12.5 FPP	10/13 (7 h)[b]
9	25	12.5	12.5 FPP	21/21 (7 h)[b]
10	0	0	25.0 FPP[c]	5/5 (6 h)
	0	0	12.5 FPP[c]	6/6 (6 h)
	0	0	25.0[c,d]	6/6 (6 h)

[a] Mevalonate depletion was performed as described in Materials and Methods.
[b] These numbers represent the sum of results of several experiments.
[c] The oocytes were soaked in progesterone (1 μg/ml).
[d] The oocytes were injected with β-glycerophosphate buffer as a control.

c-H-*ras*$^{Val-12}$ protein and either FPP, IPP, or squalene were injected into mevalonate-depleted oocytes.

Yeast and oocyte cytoplasmic extract. A soluble extract was prepared from *S. cerevisiae* JRY527 (α *ade2-101 met his 3Δ200 lys2-801 ura3-52*), a strain expressing the *DPR1/RAM1* gene, using a lysis buffer containing 10 mM Tris hydrochloride (pH 7.5), 0.1% Triton X-100, 5 mM dithiothreitol, and 4 mM EDTA. After the cells were broken with glass beads, the cells were spun at 1,000 × *g* for 2 min at 4°C in a Sorvall centrifuge. For the oocyte extract, 100 *Xenopus* oocytes were disrupted in 50 μl of the same lysis buffer but lacking Triton X-100 by drawing the sample up and down with a P200 pipetman. The crude extract was spun in a Beckman/Spinco 152 microfuge for 3 min using microfuge tubes (0.5 by 4.5 cm). Using a razor blade to slice the centrifuge tube, the cytoplasmic fraction was separated from the fat, yolk, and pigment layers.

The in vitro farnesylation assay was performed in a 25-μl reaction containing 50 mM Tris hydrochloride (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.5% Triton X-100, 20 μM c-H-*ras*$^{Val-12}$, 50 μg of cytoplasmic extract, and [^{14}C]IPP (49.7 mCi/mmol) for 2 h at 37°C. For inhibition experiments, octamer MSSKCVLS or MSSKSVLS was added to the reaction at a final concentration of 2 mM. The samples were electrophoretically separated on a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel (10). The gel was dried and exposed to X-OMAT X-ray film for 5 days at −70°C.

RESULTS

FDP is a probable substrate for the enzyme that transfers the prenyl moiety to the Ras protein. The induction of *Xenopus* oocyte maturation by c-H-*ras*$^{Val-12}$ protein can be monitored by GVBD (2) and is biologically analogous to transformation of mammalian cells and disruption of cell cycle control in *S. cerevisiae*. When *Xenopus* oocytes are injected with an unprocessed Ras protein precursor purified from *E. coli*, GVBD requires posttranslational modification of Ras protein by *Xenopus* processing enzymes in the oocyte. In mammalian and yeast cells, posttranslational processing of c-H-*ras*$^{Val-12}$ protein involves C-terminal proteolysis of the last three amino acids of Ras proteins,

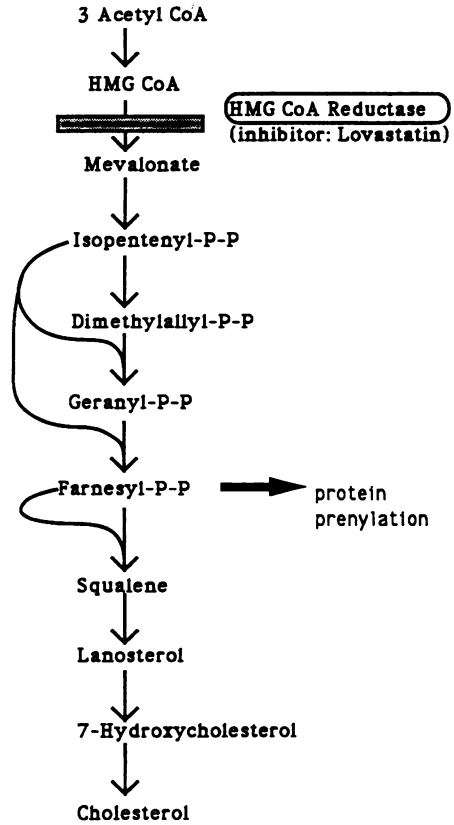

FIG. 1. Biosynthetic pathway of cholesterol and branching point for prenylation of Ras protein. The main product of mevalonate metabolism is cholesterol. The prenylated Ras protein anchors to the cytoplasmic membrane either directly or mediated by a receptor. The drug lovastatin inhibits the enzyme HMG CoA reductase. P, Phosphate.

carboxymethylation of Cys-186, and prenylation of Cys-186 (9). In *Xenopus* oocytes, the posttranslational processing of c-H-*ras*$^{Val-12}$ protein is mevalonate dependent, presumably reflecting the need for a polyisoprenoid synthesized from mevalonate (13). Protein extracts from oocytes that had been depleted of mevalonate, coinjected with [^{3}H]mevalonic acid and c-H-*ras*$^{Val-12}$ protein, and immunoprecipitated with *ras* monoclonal antibody Y13-259 were found to contain a radiolabeled protein that comigrated with the Ras protein (data not shown). This result was consistent with the observation of Casey et al. (5) in which labeling of NIH 3T3 cells with [^{3}H]mevalonic acid transfected with a *ras* oncogene also led to incorporation of radiolabel into Ras protein.

The data in Table 1 indicated that lovastatin, a drug that inhibits HMG CoA reductase does not cause GVBD by itself (Table 1, experiment 1). Injection of c-H-*ras*$^{Val-12}$ protein expressed in *E. coli* resulted in GVBD in 12 to 14 h (Table 1, experiment 2). Mevalonate depletion of oocytes blocked the action of the Ras protein (Table 1, experiment 3), but the effect of the oncogenic Ras protein could be restored by the addition of IPP (Table 1, experiments 4 and 5) or FPP (Table 1, experiment 9), both of which are intermediates of cholesterol biosynthesis (Fig. 1). The time required for GVBD was shortened to 7 h upon coinjection of c-H-*ras*$^{Val-12}$ protein with IPP or FPP (Table 1, experiments 6 and 8), whereas injection of FPP alone had no effect on the oocytes (Table 1, experiment 7). These results demonstrated that a cholesterol intermediate as far downstream as FPP can rescue the

TABLE 2. Effect of squalene and c-H-*ras*$^{Val-12}$ protein on lovastatin-treated *Xenopus* oocytes[a]

Expt no.	Amt (nl) of 2 mM lovastatin	Amt (nl) of 0.12 mM c-H-*ras*$^{Val-12}$	Amt (nl) of squalene		No. of oocytes showing GVBD/total no. of treated oocytes
			8 mM	0.8 mM	
1	0	25	0	0	5/5
2	25	25	0	0	0/4
3	0	0	12.5	0	0/6 (1)[b]
	0	0	0	12.5	0/6 (1)
4	0	25	12.5	0	5/6 (1)
	0	25	0	12.5	4/6 (2)
5	25	25	12.5	0	0/10 (2)
	25	25	0	12.5	0/11 (1)

[a] Experiment was performed as described in Materials and Methods. These numbers are the sum of data from two experiments.
[b] Values in parentheses show the number of damaged oocytes (those oocytes that died after injection).

limitation caused by lovastatin as well as shorten the time required for GVBD (see Discussion). To test whether FPP could also accelerate progesterone-induced GVBD, oocytes were injected with FPP and then soaked in progesterone (Table 1, experiment 10). FPP had no effect on progesterone-induced GVBD. Furthermore, mixed isomers of farnesol and *trans-trans*-farnesol did not have the same effect as FPP, implying a need for the high-energy bond in FPP to drive the reaction (data not shown).

Squalene, an intermediate in the cholesterol biosynthetic pathway made directly from FPP, was not able to rescue the effect of lovastatin on the transforming Ras protein (Table 2, experiment 5). Squalene by itself had no effect on the oocytes (Table 2, experiment 3), whereas coinjection of c-H-*ras*$^{Val-12}$ protein and squalene induced GVBD (Table 2, experiment 4), indicating that squalene did not inhibit the biological activity induced by the Ras protein. Since a farnesyl isoprenoid is the moiety necessary for modification of Ras proteins, the lack of rescue of Ras activation by squalene, the next intermediate after FPP suggested that FPP or a derivative other than the downstream intermediates of cholesterol biosynthesis was a substrate for the protein prenyltransferase that transfers the prenyl moiety to the Ras protein.

Prenylation of c-H-*ras*$^{Val-12}$ protein was inhibited by a peptide. The C-A-A-X motif at the carboxyl terminus of prenylated proteins appears to be at least part of the recognition determinant of the protein prenyltransferase (9). A conserved sequence motif raised the possibility that peptides modeled after that sequence may serve as competitive inhibitors of the protein prenyltransferase. The pentapeptide, containing the C-terminal sequence of c-H-*ras*$^{Val-12}$ protein, KCVLS, was coinjected at four different concentrations with c-H-*ras*$^{Val-12}$ protein. This pentapeptide did not inhibit the ability of the Ras protein to cause GVBD and by itself had no effect on oocyte maturation (Table 3).

Octapeptide MSSKCVLS, which is homologous to MSCKCVLS, the C-terminal sequence of c-H-*ras* protein, at low concentrations did not inhibit the effect of transforming Ras protein, but at higher concentrations, inhibited GVBD from going to completion (Table 4). The peptide did allow the germinal vesicle to rise to near to the top of the animal hemisphere, but upon opening of the oocyte, the nucleus was still present and GVBD did not occur (see Discussion). Injection of the octapeptide by itself had no effect on oocyte maturation.

TABLE 3. Effect of pentapeptide KCVLS and c-H-*ras*$^{Val-12}$ protein on *Xenopus* oocytes[a]

Amt (nl) of 0.12 mM c-H-*ras*$^{Val-12}$	Amt (nl) of KCVLS			No. of oocytes showing GVBD (at 10 h)/total no. of treated oocytes[b]
	10 mM	50 mM	100 mM	
25	0	0	0	4/4
0	12.5	0	0	0/2 (1)
0	0	12.5	0	0/3
0	0	0	12.5	0/3
0	0	0	25	0/3
25	12.5	0	0	6/6
25	0	12.5	0	4/4 (2)
25	0	0	12.5	6/6
25	0	0	25	6/6

[a] c-H-*ras*$^{Val-12}$ protein and pentapeptide KCVLS were coinjected into *Xenopus* oocytes.
[b] Values in parentheses are the numbers of damaged oocytes (those oocytes that died after injection).

The ability of MSSKCVLS to block complete GVBD suggested that the peptide was an inhibitor of the protein prenyltransferase responsible for prenylation of Ras proteins. Since mutations at Cys-186 of c-H-*ras* (corresponding to the Cys in MSSKCVLS) block prenylation of Ras proteins, the ability of a peptide lacking that cysteine residue four amino acids from the COOH terminus to inhibit GVBD was tested. Neither MSCKSVLS nor MSSKVVLS had an effect on GVBD (Table 4).

To test whether the inhibition of GVBD was due to the direct effect of inhibition of prenylation of the Ras protein or to some other step, we tested in vitro inhibition of the Ras prenylation in extracts from two evolutionarily distant sources. A soluble yeast extract and a cytoplasmic fraction from *Xenopus* oocytes were used to prenylate c-H-*ras*$^{Val-12}$ in the presence of [^{14}C]IPP (Fig. 2) in vitro. Addition of the octamer MSSKCVLS to the yeast extracts inhibited the prenylation reaction (Fig. 2a, lane 3), whereas the octamer MSCKSVLS did not inhibit the prenylation of c-H-*ras*$^{Val-12}$ (Fig. 2a, lane 4). Similar results were obtained with an oocyte cytoplasmic extract (Fig. 2b, lanes 2 and 3). These results indicated that the peptide containing the KCVLS consensus sequence inhibited the prenylation of Ras and that inhibition resulted in the biological block to complete GVBD. The prenylated Ras protein formed in these extracts migrated slightly faster than the unmodified protein in SDS-polyacrylamide gels, suggesting that they may be trimmed at the COOH terminus.

DISCUSSION

This work provided evidence that in *Xenopus* oocytes Ras proteins incorporate label derived from [^{3}H]mevalonic acid, as previously observed in mammalian cells (5, 9). Recent work has shown that the lipid involved in posttranslational modification of mature Ras proteins is a polyisoprenoid and is most likely farnesyl, an intermediate of sterol biosynthesis (5). Since farnesyl is derived from mevalonate, drugs such as lovastatin that interfere with HMG CoA reductase also inhibit farnesylation of Ras proteins and thereby inhibit membrane association of the protein. In *Xenopus* oocytes,

TABLE 4. Effect of octapeptides on *Xenopus* oocytes[a]

Amt (nl) of 0.12 mM c-H-*ras*$^{Val-12}$	Amt (nl) of octapeptide					No. of oocytes showing GVBD/total no. of treated oocytes (total)
	MSSKCVLS		MSSKVVLS (12.5 mM)	MSCKSVLS		
	10 mM	50 mM		25 mM	50 mM	
12.5	0	0				4/4
0	12.5	0				0/3
0	0	12.5				0/2 [1 GVBD]
0	0	25				0/3
0	0	50				0/3
12.5	12.5	0				6/6 [9 h]
12.5 (210:1)[b]	0	12.5				1/6 [5 raised GV[c]]
12.5 (420:1)[b]	0	25				[12 raised GV]
12.5 (840:1)[b]	0	50				[11 raised GV]
0			50			0/3
12.5			50			6/6
0				25	0	0/3
0				0	50	0/3
12.5				25	0	6/6
12.5				0	50	6/6

[a] The octapeptides were dissolved in buffer A, and the pH was adjusted to 7.5 with NaOH. *Xenopus* oocytes were injected with a mixture of c-H-*ras*$^{Val-12}$ protein and each of the octapeptides.

[b] Ratio of peptide to Ras.

[c] Raised GV describes a state in the oocytes in which GVBD did not go to completion and the nucleus was not broken down.

FPP can rescue the activity of Ras proteins from the inhibitory effect of lovastatin, but squalene, the next downstream intermediate in the sterol pathway made directly from FPP, could not. This result established that FPP or a derivative, was a substrate for the enzyme that attaches the prenyl group to Ras proteins. Furthermore, the observation that injected FPP, IPP, or mevalonate accelerated GVBD suggests the following three possible mechanisms. (i) The prenylation enzyme is slow, (ii) FPP is needed at some downstream process during GVBD induction, or (iii) the production of sufficient FPP required to modify the injected Ras is normally a very slow process. We have observed that the amount of incorporation of radioisotope labeling of the Ras protein was the same at 2 or 4 h after injection of [^{3}H]mevalonate (data not shown), suggesting that prenylation is fast enough. To test the second mechanism, FPP was added to oocytes induced by a different signal, progesterone; we found that progesterone-induced GVBD is not responsive to FPP (Table 1, experiment 10). Thus, the indication is that FPP may be the limiting factor in Ras-induced GVBD in oocytes.

The [^{14}C]IPP-labeled Ras proteins migrated as a single band and appeared to migrate slightly faster than the unmodified protein. At present, we cannot unambiguously distinguish whether the modified protein is proteolytically trimmed or methylated at the carboxyl terminus.

Recent evidence has shown that a significant fraction of mevalonate-labeled proteins actually contain a 20-carbon polyisoprenoid, geranylgeranyl moiety (7, 12). Due to the unavailability of geranylgeranyl diphosphate, it was not possible to test whether it too could restore GVBD by oncogenic Ras proteins of lovastatin-treated oocytes.

Whereas the pentapeptide KCVLS was not able to inhibit GVBD when coinjected with c-H-*ras*$^{Val-12}$ protein, the octapeptide MSSKCVLS did inhibit GVBD, allowing only a raised germinal vesicle, presumably by inhibiting prenylation of the Ras protein. It should be noted that inhibition of processing of the Ras protein required a 100-fold molar excess of injected competitive peptide. However, in the absence of information regarding the stability of the peptide in the oocyte or its extract, it is impossible to make any quantitative conclusions regarding the relative affinity of the processing enzyme for the substrate. On the other hand, the octapeptides MSSKVVLS and MSCKSVLS, which lacked the C-terminal Cys residue equivalent to Cys-186 in the Ras protein, had no inhibitory effect. The behavior of these peptides is consistent with earlier genetic studies in which mutations removing Cys-186 from c-H-*ras* blocked its transforming ability (14). The inhibition of prenylation by the octamer MSSKCVLS in the in vitro experiment using a soluble yeast extract or a cytoplasmic oocyte extract and c-H-*ras*$^{Val-12}$ indicated that the effect of the peptide is specific to the prenylation step. The ability of short peptides to block the processing of c-H-*ras* raised the possibility that small molecules could be found or created that can inhibit processing of Ras proteins pharmacologically.

At present, the reason for the failure of the pentapeptide KCVLS to compete for the processing of c-H-*ras* protein is unclear. A trivial reason might be that the pentapeptide is less stable than the octapeptide in the oocytes or the pentapeptide may have an incorrect conformation for preventing recognition by the protein prenyl transferase. Alternatively, the sequence or structural requirements for the enzymes that modify the Ras protein may be more complex than can be presented by a pentapeptide. The addition of the C-A-A-X motif to the carboxyl terminus of protein A is sufficient to cause prenylation of *Staphylococcus aureus* protein A, suggesting that the C-A-A-X motif is sufficient for modification (9). However, the environment of this sequence at the end of protein A may be substantially different from that of an isolated pentapeptide. Thus, these results raise

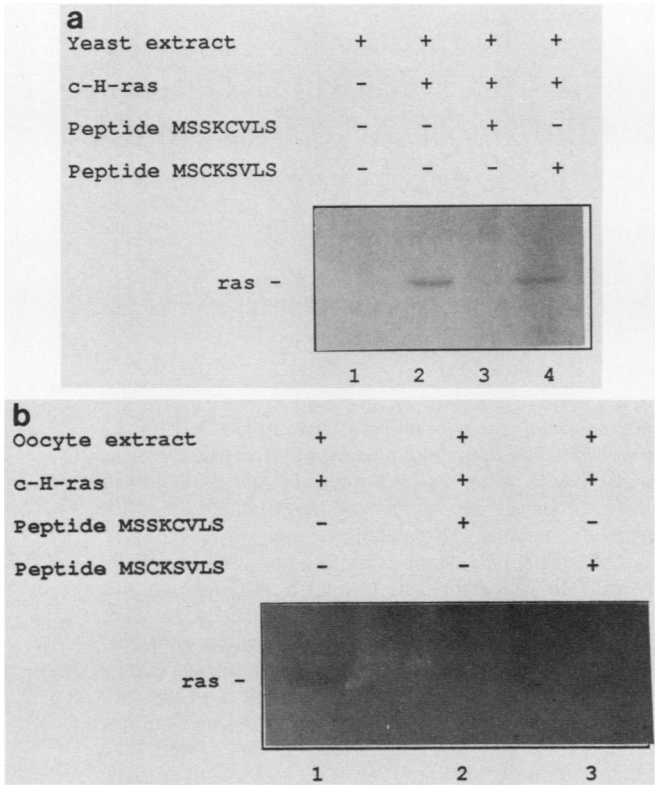

FIG. 2. Prenylation of c-H-*ras*$^{Val-12}$ in vitro in the presence of a soluble yeast extract or an oocyte cytoplasmic extract. Protein prenylation was conducted as described in Materials and Methods. After a 2-h incubation at 37°C, 25 μl of the reaction mixture was boiled for 5 min in a solution consisting of 62.5 mM Tris hydrochloride (pH 6.8), 10% glycerol, 2% SDS, and 5% β-mercaptoethanol. The products were separated on a 15% SDS-polyacrylamide gel and detected by autoradiography. (a) Prenylation with a yeast extract. Lane 1, control lane, no c-H-*ras*$^{Val-12}$; lane 2, c-H-*ras*$^{Val-12}$; lane 3, c-H-*ras*$^{Val-12}$ and 2 mM of peptide MSSKCVLS; lane 4, c-H-*ras*$^{Val-12}$ and 2 mM of peptide MSSKSVLS. (b) Prenylation with an oocyte extract. Lane 1, c-H-*ras*$^{Val-12}$; lane 2, c-H-*ras*$^{Val-12}$ and 2 mM of peptide MSSKCVLS; and lane 3, c-H-*ras*$^{Val-12}$ and 2 mM of peptide MSSKSVLS.

caution in assuming that any protein ending in a C-A-A-X motif will be prenylated.

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ADDENDUM

While this paper was under review, another paper appeared reporting the successful prenylation of Ras protein by a mammalian protein prenyl transferase and the inhibition of this reaction with peptides containing the C-A-A-X motif (Y. Reiss, J. Goldstein, M. C. Seabra, P. J. Casey, and M. S. Brown, Cell **62**:81–88, 1990).

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