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Enzymatic modification of proteins with a geranylgeranyl isoprenoid

(protein prenylation/covalent modification/GTP-binding protein)

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ABSTRACT **The prenylation of several proteins involved in oncogenesis and signal transduction plays an essential role in regulating their biological activities. Two distinct isoprenoids are known to be involved in this modification, the 15-carbon farnesyl and 20-carbon geranylgeranyl groups. Thus far, identified farnesylated proteins contain methionine or serine at the COOH terminus, while those modified by geranylgeranyl end in leucine. This report describes the characterization of an enzyme activity that transfers the geranylgeranyl group to candidate proteins. The enzyme, termed a "protein geranylgeranyltransferase," exhibits a marked preference for substrate proteins that contain leucine at the COOH terminus. In fact, the enzyme will efficiently modify a normally farnesylated protein, Ha-ras, if its COOH-terminal amino acid is switched from serine to leucine. Additional studies characterize this enzyme and suggest that it is responsible for the geranylgeranyl modification of a number of GTP-binding proteins (or their subunits) that contain a consensus prenylation sequence ending in leucine.**

The covalent modification of proteins by isoprenoid lipids (prenylation) is now recognized as a mechanism to promote membrane interactions and biological activities of these proteins (1, 2). Both farnesyl (15-carbon) and geranylgeranyl (20-carbon) isoprenoids are involved in these modifications. Known farnesylated proteins include the *ras* protooncogene products, for which prenylation is required for expression of oncogenic potential (3–5), and the nuclear protein lamin B (6, 7). The geranylgeranyl group has been found on the γ subunits (G_γ subunits) of some signal-transducing GTP-binding proteins (G proteins) (8, 9) and at least three members of the family of "small" GTP-binding proteins (10–12). This latter group includes the rap1A protein, which is of particular interest because of its ability to reverse *ras*-induced transformation of cells (13).

The current consensus sequence for prenylation includes an invariant cysteine residue, initially four residues from the COOH terminus of the protein, to which the prenyl group is attached. This region has been dubbed the "Cys-AAX motif," where the A (aliphatic) and X (initially undefined) residues specify amino acids that are removed following prenylation, freeing the COOH group of the modified cysteine residue for methylation (14, 15). The studies noted above suggest that it is the COOH-terminal residue (X) that defines which isoprenoid will be added to the protein, as methionine or serine at this position appears to dictate farnesyl addition whereas leucine signals a geranylgeranyl modification (3–12).

Since it may be possible to control the processing and biological activities of the ras and related proteins by using specific inhibitors of the prenylation machinery, there is a great deal of interest in determining both the mechanistic details of protein prenylation and the functional properties imparted by these modifications. The first step in these investigations is the isolation and characterization of the enzymes (protein prenyltransferases) that carry out protein prenylation. The first such enzyme, a protein farnesyltransferase (farnesyltransferase) capable of modifying the ras proteins, has been identified and isolated (16, 17). This enzyme has the capacity to recognize short peptides containing the Cys-AAX motif, enabling a detailed analysis of the sequence requirements for farnesylation (16, 18). However, the farnesyltransferase poorly recognizes peptides containing the Cys-AAX motif of known geranylgeranylated proteins, suggesting that a separate enzyme is responsible for this modification. In this report we identify this enzyme, termed a "protein geranylgeranyltransferase" (GerGer-transferase), and document properties of the enzyme that should greatly facilitate investigation of the role of protein prenylation in oncogenesis and signal transduction.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Proteins. The ras-Cys-Val-Leu-Leu construct (ras-CVLL; in which the Ha-ras COOH-terminal residue serine has been replaced by leucine) and rap1A construct, in expression vectors based on pXVR (19), were obtained from Channing Der (La Jolla Cancer Research Foundation, CA). The plasmids were transformed into *Escherichia coli* strain PR13Q. Protein production was induced, and extracts were prepared and separated into soluble and insoluble fractions. The recombinant proteins were purified from the insoluble fraction (R. Deloskey and S. Burk, personal communication), which contained the bulk of the ras-CVLL or rap1A, apparently in inclusion bodies. Briefly, the insoluble fraction obtained from a 1-liter culture of cells was extracted with 10 ml of 3.5 M guanidine-hydrochloride, and the extract was rapidly diluted to 500 ml with an ice-cold buffer consisting of 50 mM Tris chloride (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 3 mM $MgCl_2$, and 10 μM GDP. After a 1-hr incubation at 2°C, this mixture was centrifuged at 30,000 × *g* for 1 hr, and the supernatant was chromatographed on DEAE-Sephacel as described (16). ras-CVLL was eluted at ≈200 mM NaCl from this column, while rap1A did not adsorb and was recovered from the flow-through fraction. The proteins were identified by both SDS/PAGE analysis and GTP-binding activity. The protein pools were concentrated to ≈3 mg/ml in a Centriprep 10 concentrator, flash-frozen in multiple aliquots, and stored at −70°C.

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Abbreviations: GerGer-transferase, protein geranylgeranyltransferase; ras-CVLL, Ha-ras in which the COOH-terminal residue serine has been replaced by leucine; G protein, GTP-binding protein; G_γ subunit, γ subunit of G protein; farnesyltransferase, protein farnesyltransferase.

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The Ha-ras protein was purified from a bacterial expression system as described (16).

Purification of GerGer-Transferase. An extract was prepared from five bovine brains as described for a similar preparation from rat brains (16) and centrifuged for 90 min at 30,000 × *g*. The resulting supernatant fraction (2500 ml) was flash-frozen by pouring into liquid nitrogen and stored at −80°C until use.

Six hundred milliliters of the supernatant obtained at high speed from the bovine brain extract was chromatographed on a 5.0 × 25 cm column of DEAE-Sephacel (Pharmacia). The column, equilibrated in 50 mM Tris chloride, pH 8.0/1 mM EDTA/1 mM dithiothreitol (buffer A) containing 0.2 mM phenylmethylsulfonyl fluoride, was washed with 500 ml of the same buffer and then eluted with a gradient of 2 liters of 0–500 mM NaCl in the same buffer. Fractions of 22 ml were collected. The fractions containing the peak of GerGer-transferase activity (fractions 42–55 in Fig. 1 *Upper*) were pooled, concentrated, and chromatographed on a 5.0 × 40 cm column of AcA34 resin (Spectrum Laboratories, Houston) in buffer A containing 100 mM NaCl. Fractions of 9 ml were collected. The peak fractions from the AcA column (fractions 47–55) were pooled, concentrated to 20 ml, dialyzed against buffer A, and chromatographed on a Mono-Q HR 10/10 column (Pharmacia) equilibrated in buffer A. The column was

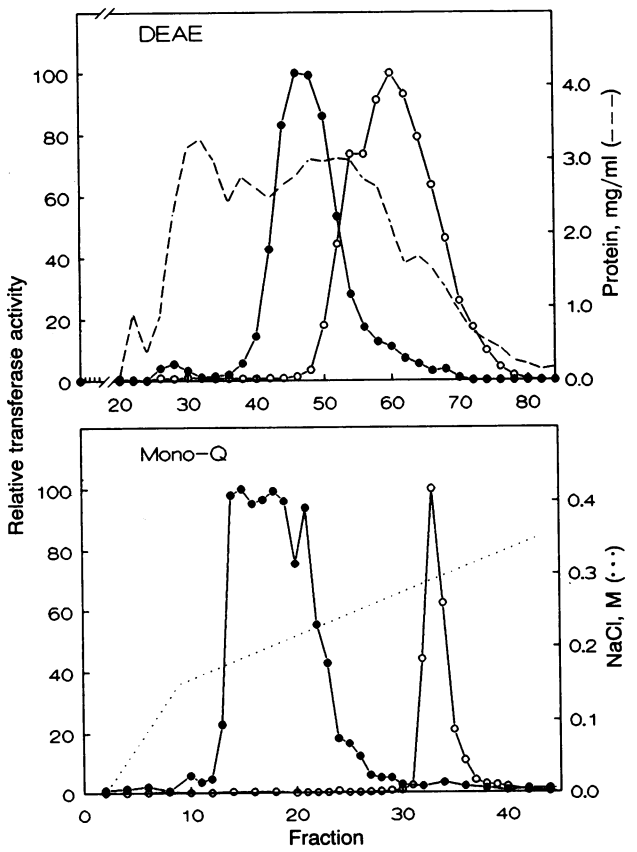

FIG. 1. Purification of GerGer-transferase. (*Upper*) DEAE chromatography. Six hundred milliliters of the supernatant from the bovine brain extract was chromatographed on DEAE-Sephacel as described. Protein prenyltransferase activities are reported relative to the peak fraction, which were 330 and 1260 units·ml^{-1} for GerGer-transferase (●) and farnesyltransferase (○), respectively. (*Lower*) Mono-Q chromatography. The peak fractions from the AcA34 column were chromatographed on a Mono-Q HR 10/10 column as described. Protein prenyltransferase activities are reported as in *Upper* and were 3500 and 3200 units·hr^{-1} for the peak fractions of GerGer-transferase (●) and farnesyltransferase (○), respectively.

washed with 10 ml of the same buffer and eluted with a 100-ml gradient of 0–400 mM NaCl in buffer A (see Fig. 1 *Lower* for gradient conditions). Fractions of 2 ml were collected. Fractions 14–22 constituting the GerGer-transferase peak were pooled, concentrated to 5 ml, flash-frozen in multiple aliquots, and stored at −80°C.

Protein Prenyltransferase Assays. Protein prenyltransferase activity was determined by quantitating the amount of ^{3}H transferred from [^{3}H]geranylgeranyl pyrophosphate or [^{3}H]farnesyl pyrophosphate into the appropriate acceptor protein (Ha-ras, ras-CVLL, or rap1A) essentially as described for farnesyltransferase assays (16). The standard reaction mixture contained the following components in a final volume of 50 μl: 50 mM Tris chloride (pH 7.7), 5 mM $MgCl_2$, 50 μM $ZnCl_2$, 2 mM dithiothreitol, 4 μM acceptor protein, 2 μM [^{3}H]geranylgeranyl pyrophosphate or [^{3}H]farnesyl pyrophosphate (at 2000 dpm/pmol), and 12 μg of resolved GerGer-transferase or 5 μg of resolved farnesyltransferase. Assays were conducted for 1 hr at 37°C. One unit of enzyme activity is defined as the amount of enzyme that transfers 1 pmol of [^{3}H]geranylgeranyl or [^{3}H]farnesyl into acid-precipitable protein per hr under the standard conditions.

Materials. Unlabeled geranylgeranyl and farnesyl pyrophosphates were synthesized by R. K. Keller, University of S. Florida, who also synthesized [1-^{3}H]geranylgeranyl pyrophosphate (8 Ci/mmol; 1 Ci = 37 GBq). [1-^{3}H]farnesyl pyrophosphate (20 Ci/mmol) was obtained from New England Nuclear. Peptides, synthesized as described (18), were dissolved in 10 mM dithiothreitol just prior to use. All peptides were purified by HPLC, and their identities were confirmed by amino acid analysis.

RESULTS

Our initial approach to identifying a GerGer-transferase activity was to screen several mammalian tissues for a heat-labile activity capable of transferring ^{3}H from [^{3}H]geranylgeranyl pyrophosphate into candidate acceptor proteins produced in bacterial expression systems. These acceptor proteins were ras-CVLL [Ha-ras protein in which the COOH-terminal amino acid was changed from serine to leucine, resulting in the same Cys-AAX sequence of the geranylgeranylated protein G25K (12)] and rap1A. Tissue extracts were prepared and separated into soluble and particulate fractions by centrifugation at 30,000 × *g*. The soluble fractions of all three tissues tested–bovine brain, bovine adrenal medulla, and rabbit liver–contained detectable GerGer-transferase activities, with the brain and adrenal medulla samples exhibiting the highest activities [5 pmol·hr^{-1}·(mg of protein)$^{-1}$]. No activity in the particulate fractions of these tissues could be reproducibly observed. GerGer-transferase activity was stimulated by both Mg^{2+} and Zn^{2+} (not shown); these cations were therefore included in all enzyme assays. Since substantial amounts of the ras-CVLL protein were produced by the bacterial expression system, this protein was used as the GerGer-transferase substrate for most studies. However, most of the experiments have been repeated with recombinant rap1A protein as the substrate, and we have not observed any appreciable difference between the two proteins in their interactions with GerGer-transferase.

GerGer-transferase was purified from the supernatant from high-speed centrifugation of a bovine brain extract by sequential chromatography on DEAE, AcA34 gel filtration, and fast protein liquid chromatography Mono-Q resins. The DEAE chromatographic step provided good resolution of GerGer-transferase and farnesyltransferase activities (Fig. 1 *Upper*); this provided direct evidence that the two protein prenyltransferase activities reside on distinct proteins. The peak of GerGer-transferase activity from the DEAE column

(Fig. 1 *Upper*) was further fractionated on the AcA34 resin, where both the GerGer-transferase and the small amount of contaminating farnesyltransferase comigrated (not shown). However, this remaining farnesyltransferase activity was completely resolved from GerGer-transferase by chromatography on Mono-Q resin (Fig. 1 *Lower*). This Mono-Q-purified material ("resolved GerGer-transferase") was used for all subsequent studies. This entire purification procedure has been repeated three times and consistently results in a preparation of resolved GerGer-transferase with a specific activity of 500–700 pmol·hr^{-1}·(mg of protein)$^{-1}$, representing a 100- to 140-fold increase over the starting material, with overall yields from 50% to 60%. The enzyme displays Michaelis–Menten-type kinetics toward the prenyl substrate, with an apparent K_m of 0.3 μM for geranylgeranyl pyrophosphate, and has a pH optimum in the slightly basic range, with maximal activity being observed from pH 7.5 to pH 8.5 (not shown).

Mono-Q chromatography of the farnesyltransferase pool obtained from the DEAE column (Figure 1 *Upper*, fractions 56–66), when performed under identical conditions to those used for GerGer-transferase (Fig. 1 *Lower*), resulted in a complete separation of this activity from GerGer-transferase (not shown). This "resolved farnesyltransferase" exhibited a specific activity of 3000 pmol·hr^{-1}·(mg of protein)$^{-1}$ and was used in studies described below comparing the specificities of the two protein prenyltransferases.

Under our standard conditions, the resolved GerGer-transferase modified both the ras-CVLL and rap1A proteins, but apparently not Ha-ras, in reactions dependent on both the recombinant protein and metal ions (Fig. 2). Chemical analysis of the prenyl group attached to the acceptor proteins in these assays (21) verified that it was the geranylgeranyl group (not shown).

For a further analysis of the ability of the resolved GerGer-transferase to discriminate between Ha-ras and ras-CVLL, which differ in only their COOH-terminal residue, we examined the protein substrate dependence of both of the resolved protein prenyltransferases (Fig. 3). Consistent with the results in Fig. 2, ras-CVLL served as a substrate for the

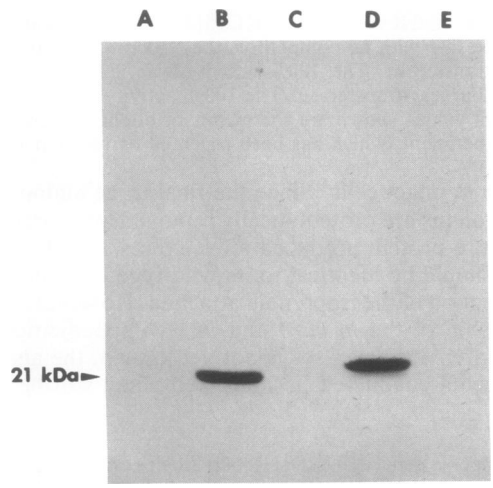

FIG. 2. Modification of recombinant proteins by resolved GerGer-transferase. Reactions were carried out as described and terminated by the addition of Laemmli sample buffer (20). Following electrophoresis through SDS/13% polyacrylamide, the gel was stained to identify markers and treated with EN^{3}HANCE (DuPont–NEN). The dried gel was exposed to Kodak AX-5 film for 36 hr at −80°C. Lanes: A, no recombinant protein was added to the reaction mixture; B–D, reaction mixtures contained 4 μM each of ras-CVLL, Ha-ras, and rap1A, respectively; E, reaction mixture contained 4 μM ras-CVLL plus an additional 10 mM EDTA to chelate metal ions (Mg^{2+} and Zn^{2+}).

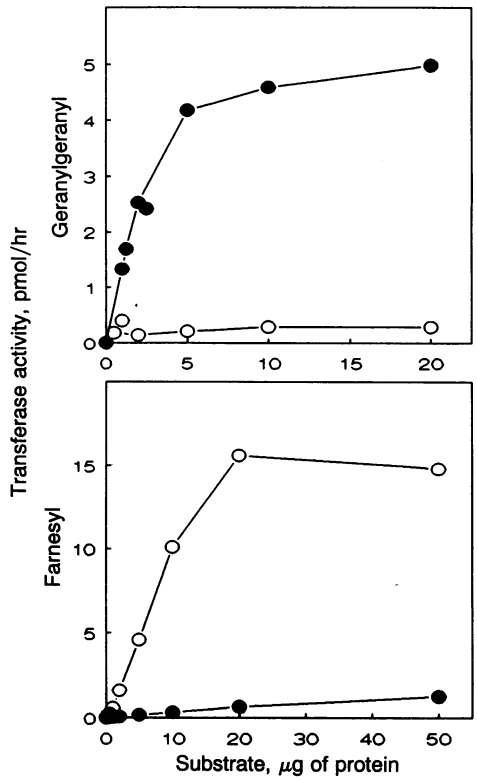

FIG. 3. Dependence of protein prenyltransferase activity on protein substrate concentration. Assays were conducted as described except that each reaction mixture contained the indicated concentration of substrate protein. (*Upper*) GerGer-transferase. (*Lower*) Farnesyltransferase. ●, ras-CVLL; ○, Ha-ras.

GerGer-transferase with an apparent K_m of 1.5 μM, while substrate activity with Ha-ras was barely detectable (Fig. 3 *Upper*). Essentially converse results were obtained with the farnesyltransferase, which accepted Ha-ras as a substrate with an apparent K_m of 8 μM, while activity with the ras-CVLL protein was very poor (Fig. 3 *Lower*).

A more detailed examination of the substrate specificities of the two protein prenyltransferases is shown in Fig. 4, where each enzyme was assayed with the four possible combinations of protein and prenyl substrates. As expected from the results described above, each protein prenyltransferase shows a marked preference for its "correct" substrates (GerGer-transferase, ras-CVLL and geranylgeranyl pyrophosphate; farnesyltransferase, Ha-ras and farnesyl pyrophosphate), although there was a small, but detectable, activity in some of the other conditions.

Like the farnesyltransferase, the resolved GerGer-transferase has the capacity to recognize short peptides that encompass the Cys-AAX prenylation consensus sequence (Fig. 5 *Upper*). In particular, we have studied the decapeptide corresponding to the COOH terminus of the brain G_γ subunit, synthesized with a tyrosine residue at the NH_2 terminus (Tyr-Arg-Glu-Lys-Lys-Phe-Phe-Cys-Ala-Ile-Leu) to facilitate future studies, and the undecapeptide corresponding to the COOH terminus of the small G protein, G25K (Glu-Pro-Lys-Lys-Ser-Arg-Arg-Cys-Val-Leu-Leu). For comparison, the decapeptide of the COOH-terminal portion of a known farnesylated protein (3), Ki-ras (Lys-Lys-Ser-Lys-Thr-Lys-Cys-Val-Ile-Met), was also analyzed. While the G_γ and G25K peptides competed for modification of ras-CVLL with EC_{50} values of 1 μM and 3 μM, respectively, the Ki-ras peptide was much less effective (EC_{50} > 30 μM). Parallel studies with the resolved farnesyltransferase showed, as expected (16, 18), a reversed specificity, with the

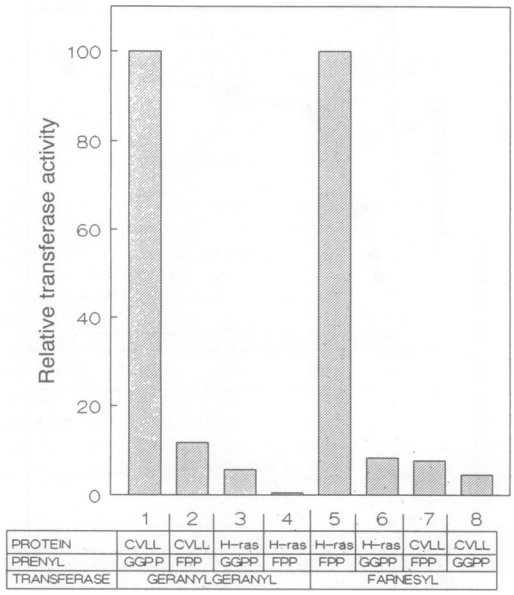

FIG. 4. Specificities of protein prenyltransferases. Assays were conducted as described. The enzyme, recombinant protein (4 μM), and prenyl pyrophosphate (2 μM) included in each condition are listed below the appropriate lanes. Activity values are reported relative to the corresponding assay containing both "correct" substrates [GerGer-transferase, geranylgeranyl pyrophosphate (GGPP) and ras-CVLL (CVLL); farnesyltransferase, Ha-ras, and farnesyl pyrophosphate (FPP)]. For GerGer-transferase (lane 1), this was 5.6 units per assay, and for farnesyltransferase (lane 5), it was 6.7 units per assay. All values are means of duplicate points from a single experiment, which has been repeated twice.

Ki-ras peptide being a very potent inhibitor of the reaction ($EC_{50} \approx 0.3$ μM) and the G_γ and G25K peptides being essentially inert at the concentrations tested. These results, coupled with the observation that the GerGer-transferase we are studying efficiently modifies the recombinant rap1A protein, strongly suggest that this enzyme is responsible for the geranylgeranyl modification of the small G proteins and G_γ subunits that contain leucine at the COOH terminus. In fact, we have recently shown that this enzyme exhibits activity toward other small G proteins with this motif, including the rac1 (22) and rap2b (L. Molina, J.F.M., and P.J.C., unpublished observations) proteins. Additionally, a similar if not identical enzyme recently described exhibits activity toward the rhoA protein (23).

DISCUSSION

The observations reported here will have an important impact on studies involving the role of protein prenylation in oncogenesis and signal transduction. The availability of the GerGer-transferase will allow an analysis of its mechanism of action and facilitate discovery and development of inhibitors specific to each class (farnesyl and geranylgeranyl) of protein prenyltransferase. This is especially important given the increasing interest in such inhibitors as possible therapeutic agents in the treatment of human cancers (24, 25). Furthermore, comparisons of the structural relationships of the two classes of protein prenyltransferases have already begun (26) and have revealed that they share a highly related, if not identical, subunit.

These results also underscore the importance of the COOH-terminal residue of prenylated proteins in determining which isoprenoid will be attached. Thus, it appears that a single amino acid change is all that will be required in cDNA expression studies designed to examine the influence of isoprenoid chain length on prenyl protein targeting and

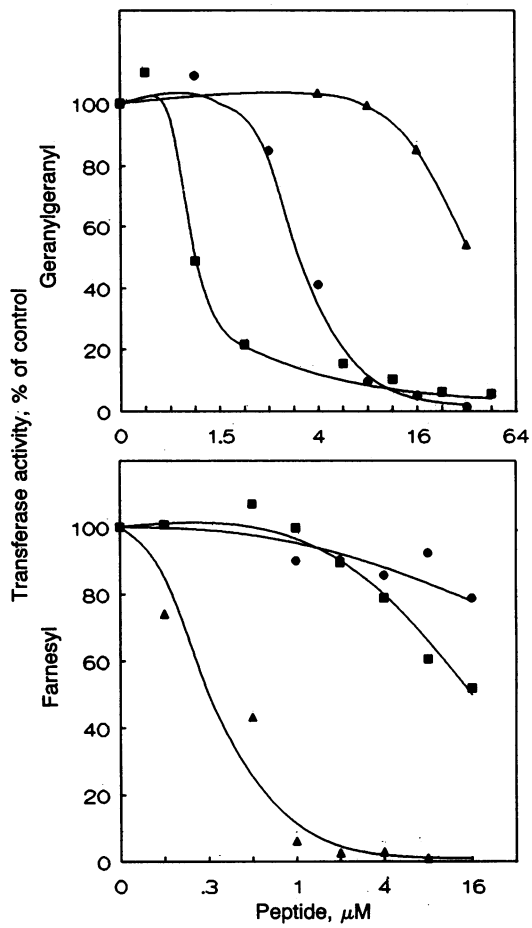

FIG. 5. Inhibition of protein prenyltransferases by Cys-AAX-containing peptides. Reactions were carried out as described except that in the GerGer-transferase assays (*Upper*), the concentration of substrate protein (ras-CVLL) was reduced to 1 μM and all reaction mixtures contained the indicated concentration of competitor peptide. The peptides used were those corresponding to the COOH-terminal sequences of G_γ (YREKKFFCAIL; ■), G25K (EPKKSRRCVLL; ●), and K-ras (KKSKTKCVIM; ▲). Activities are reported relative to that with no competitor (the "100%" activity). (*Upper*) GerGer-transferase. The 100% activity was 2.3 units per assay. (*Lower*) Farnesyltransferase. The 100% activity was 7.5 units per assay. All values shown are the means of duplicate assays from a single experiment, which has been repeated two to four times.

function in intact cells. Since the final three amino acids of these proteins are proteolytically removed after prenylation, the mature protein produced after expression of an altered cDNA should be identical to its wild-type counterpart with the exception of the isoprenoid attached. However, since the relationship of the *in vitro* and *in vivo* specificities of the protein prenyltransferases is not yet known, the analysis of experiments conducted in intact cells may still be complicated.

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