Post-translational regulation of mevalonate kinase by intermediates of the cholesterol and nonsterol isoprene biosynthetic pathways

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Abstract To assess the potential for feedback inhibition by isoprene intermediates in the cholesterol and nonsterol isoprene biosynthetic pathway, we expressed human cDNAs encoding mevalonate kinase (MKase), phosphomevalonate kinase (PMKase), and mevalonate diphosphate decarboxylase (MDDase) as fusion proteins in *Escherichia coli* DH5α, and purified these proteins by affinity chromatography. Several phosphorylated and non-phosphorylated isoprenes were analyzed as inhibitors of the enzymes using a standard spectrophotometric assay. Of the three proteins, only MKase was inhibited through competitive interaction at the ATP-binding site. The intermediates studied (and their relative inhibitory capacity) were: geranylgeranyl-diphosphate (GGPP, C_{20}) > farnesyl-diphosphate (FPP, C_{15}) > geranyl-diphosphate (GPP, C_{10}) > isopentenyl-diphosphate (IPP, C_5) ≥ 3,3-dimethylallyl-diphosphate (DMAPP, C_5) > farnesol (C_{15}) > dolichol-phosphate (DP, C_{80-100}). Mevalonate-diphosphate, geraniol, and dolichol were not inhibitors. Our data further define the spectrum of physiologic inhibitors of MKase, and provide the first evidence for feedback inhibition of MKase by a nonsterol isoprene produced by the branched pathway, dolichol-phosphate. These results provide additional evidence that MKase may occupy a central regulatory role in the control of cholesterol and nonsterol isoprene biosynthesis.—**Hinson, D. D., K. L. Chambliss, M. J. Toth, R. D. Tanaka, and K. M. Gibson.** Post-translational regulation of mevalonate kinase by intermediates of the cholesterol and nonsterol isoprene biosynthetic pathways. *J. Lipid Res.* 1997. **38:** 2216–2223.

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Mevalonate, the product of the reaction catalyzed by 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, is a key intermediate in the de novo synthesis of sterols and nonsterol isoprenes (**Fig. 1**). In mammalian cells, the majority of mevalonate is converted to cholesterol, a portion of which is converted to steroid hormones, bile acids, and vitamin D. Mevalonate is also converted into other important intermediates, including *1*) dolichols, which act as carriers in the assembly of carbohydrate chains in glycoproteins; *2*) ubiquinones, which participate in electron transport; *3*) isopentenylated transfer RNAs, which are important for protein synthesis; and *4*) farnesylated and geranylgeranylated proteins, which are membrane associated and appear to be involved in intracellular signaling (1).

The principal regulatory point in the pathway of cholesterol and nonsterol isoprene biosynthesis is HMG-CoA reductase, a membrane-bound enzyme of the endoplasmic reticulum. In mammalian cells, regulatory mechanisms adjust the activity of HMG-CoA reductase over a range of several 100-fold, assuring the production of sufficient mevalonate for conversion into essential sterol and nonsterol products (1). The principal regulatory mechanisms that maintain mevalonate homeostasis include sterol-mediated feedback repression of transcription of the genes encoding HMG-CoA synthase and reductase, the low-density lipoprotein (LDL) receptor, squalene synthase, and farnesyl diphosphate synthase. In addition, HMG-CoA reductase is subject to

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; MKase, mevalonate kinase; PMKase, phosphomevalonate kinase; MDDase, mevalonate diphosphate decarboxylase; LDL, low density lipoprotein; K_i, inhibition constant; K_m, Michaelis constant; $K_m$app, apparent Michaelis constant; PBS, phosphate-buffered saline; SDS, sodium-dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; M-5-P, R-mevalonate 5-phosphate; M-5-PP, R-mevalonate 5-diphosphate; GGPP, geranylgeranyl diphosphate; IPP, isopentenyl-diphosphate; DMAPP, 3,3-dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; DP, dolichol-phosphate; IPTG, isopropylthio-β-galactoside.

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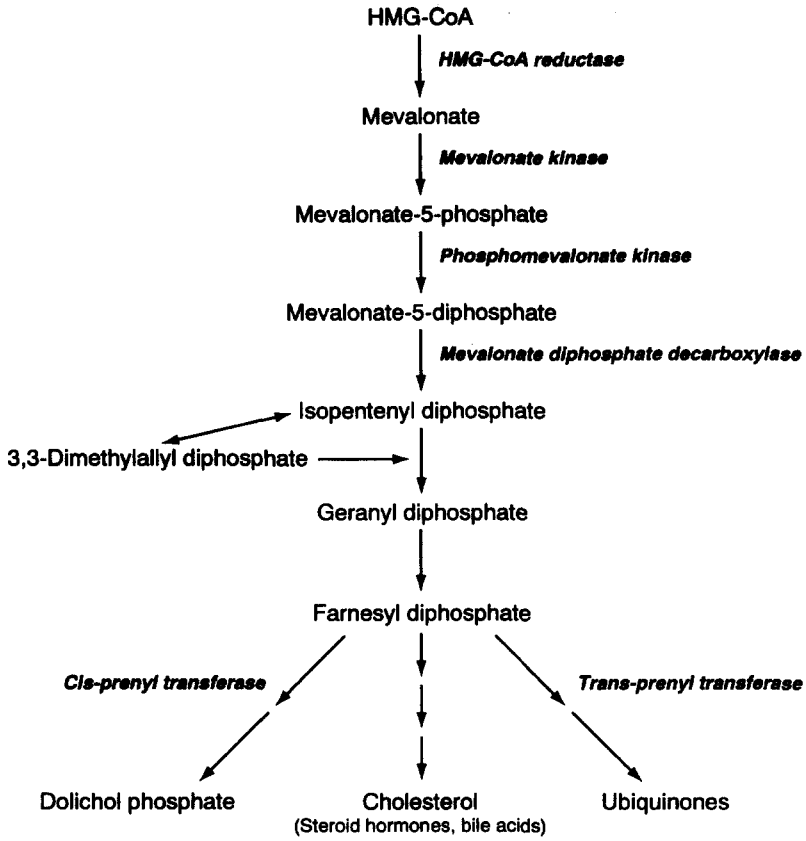

Fig. 1. The pathway of cholesterol and nonsterol isoprenoid biosynthesis in mammalian cells (not all steps are shown).

post-transcriptional control in response to sterol and other factors (1).

Recent evidence indicates that mevalonate kinase (MKase) may play a role in pathway regulation. In animals, the activities of MKase, phosphomevalonate kinase (PMKase), and mevalonate diphosphate decarboxylase (MDDase) in liver respond to dietary supplementation with cholesterol and cholesterol sequestering agents (2). For MKase, this regulation occurs at the transcriptional level, as we have recently shown that the MKase promoter contains a new version of the sterol-responsive element previously identified in the promoters of the HMG-CoA reductase and LDL-receptor genes (3, 4). In addition, MKase is subject to post-translational regulation via competitive inhibition by geranyl-diphosphate (GPP) and farnesyl-diphosphate (FPP) at the ATP binding site (5, 6).

In the pathway of cholesterol and nonsterol isoprene biosynthesis, mevalonate is converted to isopentenyl-diphosphate (IPP) by a unique sequence of three ATP-dependent enzymes, MKase, PMKase, and MDDase. Earlier work demonstrated that MKase was subject to competitive inhibition by geranyl- and farnesyl-diphosphates at the ATP binding site (5, 6). As all three cDNAs that encode these proteins are now available, we sought to develop expression plasmids for these three proteins in order to obtain purified recombinant proteins for further kinetic analysis. Our objectives were to further define the nature of the physiologic isoprene inhibitors that regulate MKase activity at the protein level, and to assess the possibility that PMKase and MDDase were susceptible to similar inhibition. The present report summarizes the results of these studies.

EXPERIMENTAL PROCEDURES

Reagents

Competent *E. coli* DH5α cells were purchased from GIBCO-BRL. Glutathione Sepharose 4B and pGEX-4T vectors 1 and 3 were obtained from Pharmacia. The human MKase cDNA was a gift from Bristol Myers Squibb Pharmaceutical Corporation (Princeton, NJ). The human MDDase cDNA was a gift from Novartis Corporation (Summit, NJ). R,S-mevalonate, ATP, and all phosphorylated and non-phosphorylated isoprenes were

purchased from Sigma. Purity of isoprenes was assessed by thin-layer chromatography with localization by iodine staining (7). With the exception of DP, dolichol, geraniol, and farnesol, all phosphorylated isoprenes were supplied as solutions in 7:3 methanol/10 mM NH_4OH, which was not inhibitory to any of the recombinant proteins at the volumes used. Dolichol and DP were supplied in 2:1 chloroform–methanol or carbon tetrachloride. Before use, these solvents were removed under gentle nitrogen purge, and the long-chain isoprenes were taken up in 50:50 dimethyl sulfoxide/50 mM MOPS-KOH buffer, pH 7.0. To avoid degradation, all isoprenes were stored during, and in between, usage in light-restrictive containers under nitrogen atmosphere. Geraniol and farnesol were diluted to the appropriate concentration in dimethyl sulfoxide. R-mevalonate 5-phosphate and R-mevalonate 5-diphosphate were prepared using the recombinant purified proteins and purified by affinity chromatography as described (8, 9). All other reagents were of the highest purity obtainable.

Expression of human MKase, PMKase, and MDDase

The human cDNA encoding MKase was ligated into the pGEX-4T-3 at the EcoR I site, and transformed into competent DH5α cells. Cells grown for 2 h in TB (Terrific Broth) medium containing ampicillin (10) were induced with 1 mM IPTG, followed by growth for 72 h at 28°C. Bacteria were isolated by centrifugation at 4°C, washed in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), and resuspended in PBS supplemented with 1% 2-mercaptoethanol. Bacteria were ruptured by sonication while maintained in an ice-bath (4 W total, 50% output, 3 min) with cooling after each minute of sonication. After rupture, the cell extracts were supplemented with 1% Triton X-100 with gentle agitation for 30 min at room temperature. Cell debris was removed by centrifugation at 10,000 *g* for 15 min. PMKase and MDDase were expressed using similar methods, with both cDNAs in the pGEX-4T-1 vector (10, 11).

Affinity purification of recombinant enzymes

Recombinant human MKase, PMKase and MDDase, expressed as the fusion proteins with glutathione *S*-transferase, were isolated by chromatography on glutathione Sepharose beads. Columns of glutathione Sepharose were prepared in disposable polypropylene columns (10 ml total volume) after suspension of the resin in cold PBS. Clarified bacterial sonicates were applied to the column and allowed to elute with gravity. The void volume after application was reapplied to the column a second time. The columns were washed with 10 bed volumes of cold PBS. The fusion proteins were eluted with glutathione elution buffer supplied by the manufacturer (Pharmacia). Eluted protein was concentrated using Centricon 10 membranes. In certain instances, site-specific cleavage of the MKase fusion protein was carried out while the fusion protein was adsorbed onto the affinity columns. For cleavage, 500 U of thrombin (Sigma) was dissolved in 0.5 ml of PBS. For each ml bed volume used, 1 ml of a 20:1 dilution of thrombin solution in PBS was applied to the column and allowed to enter the resin bed. The solution was incubated overnight at room temperature. MKase was eluted from the column using PBS supplemented with 1% 2-mercaptoethanol. Thrombin, in the concentration used, did not inhibit MKase activity. SDS-polyacrylamide gel electrophoresis was performed on 8% gels as described using Commassie staining for band visualization (6).

Enzyme assays

MKase, PMKase, and MDDase were assayed spectrophotometrically with a Perkin-Elmer 552A UV/VIS dual beam spectrophotometer fitted with strip-chart recorder (6, 10). The final assay volume was 1 ml, except for analyses using DP and dolichol, with final assay volume of 0.2 ml. The final reaction contained: 50 mM MOPS-KOH, pH 7.0, 6 mM $MgCl_2$, 10 mM KF, 5 mM ATP, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 10 U each of pyruvate kinase/lactate dehydrogenase, differing amounts of R,S-mevalonic acid, R-mevalonate 5-phosphate or R-mevalonate 5-diphosphate (depending on the enzyme under study) and varying amounts of recombinant enzyme. Linearity of reaction velocities with respect to protein content was assessed for all preparations of recombinant enzyme. Enzyme assays were performed at 25°C. For analyses with MKase, the protein content of enzyme reactions was 0.3–0.5 μg of purified recombinant enzyme; for PMKase, the standard assay used 3–5 μg purified protein, and for MDDase standard reaction mixtures used 5–10 μg of recombinant enzyme. For routine analyses, substrate concentrations (R,S-mevalonate, R-mevalonate 5-phosphate, or R-mevalonate 5-diphosphate) were 2 mM, 0.2 mM, and 0.1 mM, respectively. Protein concentration in assays were adjusted such that less than 10% of substrate was consumed during the course of the initial reaction.

In the spectrophotometric assay, ADP produced in the MKase, PMKase, or MDDase reactions is coupled to pyruvate kinase, which converts phosphoenolpyruvate to pyruvate; pyruvate thus formed is utilized by lactate dehydrogenase to oxidize NADH to NAD^+ with production of lactate. In this way, ADP production is stoichiometrically related to NADH consumption. All reaction contents were combined and equilibrated except for substrate, and the background consumption of NADH

was monitored until a stable baseline was reached. Reactions were then initiated by addition of substrate, and the decrease in absorbance was determined for 1–2 min, using the extinction coefficient for NADH of 6.22 mM^{-1} cm^{-1}. For assay of MKase in extracts of *E. coli*, we used a standard radiometric assay using [2-^{14}C]R,S-mevalonate, with separation of unreacted substrate from phosphorylated products by thin-layer chromatography (12).

Kinetic determinations

$K_m$app for R,S-mevalonate, R-mevalonate 5-phosphate, and R-mevalonate 5-diphosphate were determined using a fixed ATP concentration of 5 mM. Similarly, $K_m$app for ATP for all enzymes was determined using a fixed, saturating concentration of the appropriate substrate as described above. Individual K_i values for phosphorylated and non-phosphorylated isoprenes were determined using fixed, saturating concentrations of the appropriate substrates and concentrations of ATP ranging from 0.16 to 5 mM. Each concentration of ATP was run at least in duplicate with each concentration of inhibitor, and mean velocities were utilized in final determination of kinetic constants. From two to four concentrations of each inhibitor were used in reactions with varying levels of ATP. The resulting K_m values obtained were plotted versus the concentration of inhibitor to determine the K_i value. All calculations of kinetic parameters were derived using a computer-assisted program based upon standard equations (13). Because the DP preparation utilized was a mixture of isoprene lengths C_{80}–C_{105}, kinetic data were plotted as a function of the μg quantities of DP used. The molecular weight used for estimation of DP concentration was an average of the chain-lengths supplied, or 1358 g/mole.

RESULTS

Expression of human MKase, PMKase, and MDDase

Using glutathione-Sepharose affinity chromatography, we obtained highly enriched recombinant human MKase, PMKase, and MDDase using *E. coli* DH5α as host (**Fig. 2**). As the fusion proteins with glutathione *S*-transferase (approximate molecular weight 32 kDa), we observed essentially a single Coomassie-staining band after polyacrylamide gel electrophoresis for each recombinant fusion protein. Estimated molecular weights were: MKase 74.1 kDa, PMKase 51.3 kDa, and MDDase 77.6 kDa, with unfused glutathione *S*-transferase migrating at an estimated MW consistent with 31.6 kDa. These data yielded non-fusion protein molecular weights of 42.5, 19.7, and 46.0 kDa, respectively (expected 42, 21.9, and 43 kDa, respectively). The major band in the preparation of MDDase migrated with an estimated molecular weight of 32 kDa, corresponding to unfused glutathione *S*-transferase.

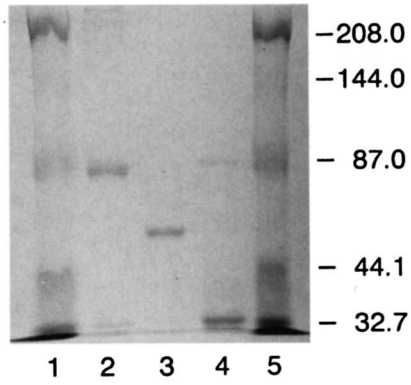

Fig. 2. SDS-PAGE analysis of recombinant human ATP-dependent mevalonate catabolic enzymes. The fusion proteins of mevalonate kinase (MKase), phosphomevalonate kinase (PMKase), and mevalonate diphosphate decarboxylase (MDDase) with glutathione *S*-transferase were purified by glutathione-Sepharose affinity chromatography. Lane contents of the gel were: 1 and 5, molecular weight standards; 2, MKase; 3, PMKase; 4, MDDase. Molecular weights of protein standards are depicted on the y-axes.

Kinetic characterization of recombinant MKase, PMKase, and MDDase

$K_m$app values for the appropriate mevalonate derivatives were 150, 25, and approximately 7 μM for MKase, PMKase, and MDDase, respectively (**Table 1**). The same values for substrate ATP for the three proteins ranged from 0.26 to 0.44 mM, holding the respective mevalonate substrate (free mevalonate or the appropriate phosphorylated substrate) at saturating concentration.

With the recombinant proteins isolated, we sought to characterize the capacity of various phosphorylated and non-phosphorylated isoprenes to serve as inhibitors of these enzyme reactions (Table 1). Earlier results had demonstrated that MKase isolated from porcine liver could be competitively inhibited by FPP and GPP (5, 14). We reproduced these findings with recombinant MKase, but substantially extended these earlier findings. We found MKase susceptible to competitive inhibition with respect to ATP by a variety of compounds (Table 1). The intermediates studied (and their relative inhibitory capacity) were: geranylgeranyl-diphosphate (GGPP, C_{20}) > farnesyl-diphosphate (FPP, C_{15}) > geranyl-diphosphate (GPP, C_{10}) > isopentenyl-diphosphate (IPP, C_5) ≥ 3,3-dimethylallyl-diphosphate (DMAPP, C_5) > farnesol (C_{15}) > dolichol-phosphate (DP, C_{80-100}) (see **Fig. 3** for representative data). Mevalonate-diphosphate, geraniol, dolichol, and inorganic pyrophos-

TABLE 1. Kinetic constants of recombinant human mevalonate kinase (MKase), phosphomevalonate kinase (PMKase), and mevalonate diphosphate decarboxylase (MDDase) purified from bacterial extracts

Intermediate	Constant	MKase	PMKase	MDDase
R,S-melavonate	$K_m$app	150 ± 23 μM (n = 4)[a]	—	—
R-melavonate-5-P	$K_m$app	—	25 ± 6 μM (n = 3)	—
R-melavonate-5-PP[b]	K_i or $K_m$app	NI (to 84 μM)	—	7.4 μM
ATP	$K_m$app	0.44 ± 0.19 mM (n = 5)	0.26 ± 0.03 mM (n = 3)	0.32 mM
Isopentenyl-PP	K_i	16 μM	NI (to 64 μM)	—
3,3-Dimethylallyl-PP	K_i	20 μM	NI (to 61 μM)	NI (to 61 μM)
Geranyl-PP	K_i	116 nM	NI (to 52 μM)	NI (to 52 μM)
Farnesyl-PP	K_i	104 ± 44 nM (n = 5)	NI (to 44 μM)	NI (to 44 μM)
Geranylgeranyl-PP	K_i	59 ± 11 nM (n = 6)	—	—
Dolichol-P	K_i	83 μM[c]	—	—
Farnesol	K_i	72 μM	—	—
Geraniol	K_i	NI (to 226 μM)	—	—
Dolichol	K_i	NI (to 0.68 mM)	—	—

All kinetic constants were the mean of at least duplicate (and up to six) determinations. In some instances, studies on mevalonate kinase used the thrombin-cleaved fusion protein (glutathione-*S* transferase fused to mevalonate kinase). Thrombin was not removed from these preparations, and was not an inhibitor of mevalonate kinase activity. Kinetic constants determined with either mevalonate kinase or the mevalonate kinase fusion protein did not differ significantly. All studies with phosphomevalonate kinase and mevalonate diphosphate decarboxylase were performed using the fusion protein. P, phosphate; PP, diphosphate; ATP, adenosine triphosphate; $K_m$app, apparent K_m (i.e., with second substrate maintained at a single constant concentration); K_i, inhibition constant; NI, not inhibitory up to the concentration indicated.

[a] ± Standard error of the mean.

[b] In this instance, mevalonate 5-diphosphate was tested both as substrate of mevalonate diphosphate decarboxylase and as inhibitor of mevalonate kinase.

[c] Concentrations estimated from assumed mean molecular weight of 1358 g/mole.

phate (data not shown) were not inhibitory toward MKase. Commercial unavailability of dolichol-PP, and lability of the synthesized compound, prevented studies of this intermediate as a potential inhibitor of MKase.

To further assess the potential physiologic significance of DP inhibition of MKase, we studied the capacity of DP to inhibit MKase activity in extracts of bacteria expressing human MKase. In these analyses, we used a saturating concentration of R,S-mevalonate and a concentration of ATP that approximated the K_m concentration (0.5 mM) for ATP. As these studies were performed in crude bacterial extracts, the spectrophotometric assay based upon NADH consumption was not feasible; rather, a radiometric procedure was used (12). Control assays contained identical concentrations of dolichol alone and demonstrated a mean activity of 817 ± 126 pmol/min per mg protein (±SEM, n = 5 determinations on separate bacterial pellets). The same activities were 430 ± 24 pmol/min per mg protein (±SEM, n = 5) for assays containing 198 μM DP, and 345 ± 38 pmol/min per mg protein (± SEM, n = 4) for assays containing 396 μM DP. The latter two mean activities were significantly different from assays containing only dolichol ($P < 0.05$, two-tailed t test).

The availability of human cDNAs encoding the next two enzymes in the pathway, PMKase and MDDase, enabled the opportunity to assess the possibility that these enzymes were susceptible to comparable inhibition by phosphorylated and non-phosphorylated isoprenes (Table 1). At concentrations which almost completely inactivated MKase activity, a variety of compounds, including IPP, DMAPP, FPP, and GPP, were not inhibitory toward PMKase and MDDase (Table 1).

DISCUSSION

In this comparative study, we expressed the human cDNAs encoding MKase, PMKase, and MDDase in *E. coli* using the pGEX expression system (Fig. 2). The resultant fusion proteins offered a simple and rapid method for affinity purification of the recombinant proteins. To determine whether these proteins demonstrated properties comparable to their mammalian counterparts, we determined apparent K_m values for ATP and the respective mevalonate substrate (Table 1).

In earlier studies using a radiometric assay and crude extracts of cultured cells as source of MKase, we measured $K_m$app values of 0.23–0.53 mM for ATP (12). Porcine and chicken liver MKase showed similar $K_m$app values of 0.25–0.68 mM for ATP and 10–50 μM for R,S-mevalonate. Tanaka and coworkers (6) presented a K_m of approximately 271 μM for substrate R,S-melavonate using MKase purified from rat liver. These values were consistent with those reported here using recombinant human MKase (Table 1). Bazaes and coworkers (15) determined true K_m values for purified porcine PMKase of 75 μM and 0.45 mM for mevalonate 5-phosphate and ATP, respectively, which were somewhat higher than the $K_m$app values we determined for the human recombinant protein (Table 1). K_m values of 0.45–0.60 mM

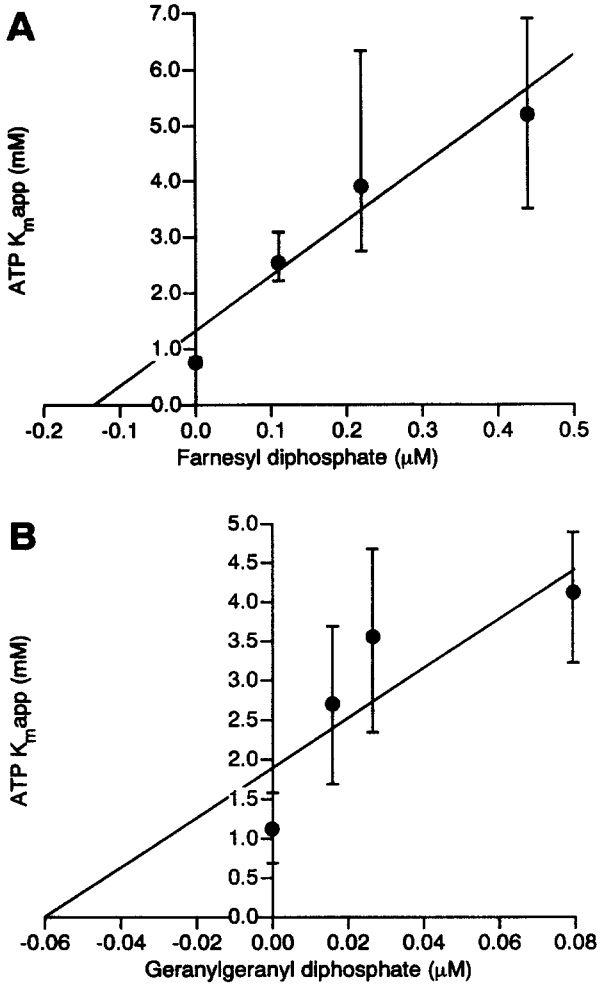

Fig. 3. Inhibition of recombinant human mevalonate kinase fusion protein activity by farnesyl-diphosphate (A) and geranylgeranyl-diphosphate (B). For each compound, $K_m$app for ATP was determined in the presence of either no inhibitor or three fixed concentrations (x-axis). As only the x intercept ($-1/K_m$) was altered and not the $1/V_{max}$ (data not shown), these isoprenes inhibited mevalonate kinase by binding competitively at the ATP-binding site on the enzyme. The graphs depict the plots of $K_m$app values for ATP determined in the presence or absence of inhibitor versus the respective inhibitor concentrations. Error bars indicate variance in the range of values obtained for farnesyl-diphosphate (n = 5 analyses at each point) and geranylgeranyl-diphosphate (n = 6 analyses at each data point). The x intercept of the lines represent the $-K_i$ value (13).

and 2–12 μM for ATP and mevalonate 5-diphosphate, respectively, were reported for MDDase isolated from porcine liver and the purified recombinant human protein (9, 11). These values were comparable to our results for the recombinant human MDDase (Table 1).

As shown in Table 1, we found MKase, but not PMKase and MDDase, susceptible to competitive inhibition by a wide variety of phosphorylated and non-phosphorylated isoprene intermediates of the cholesterol and nonsterol isoprene biosynthetic pathway. In 1968, Dorsey and Porter (5) showed that porcine liver MKase was subject to competitive inhibition by GPP and FPP at the ATP-binding site. Tanaka and coworkers (6) corroborated these findings using purified rat liver MKase. Both groups found K_i values of 1.2–2.7 μM for GPP and FPP. In the current study, the K_i values were considerably lower at 104–116 nM (Table 1). The K_i determined for GPP (Table 1) approximated 10% of the K_m value (1 μM) for this compound in the rat FPP synthase reaction, and was considerably below the approximate K_m of 25 μM for GPP in the *trans*-prenyltransferase reaction in rat liver microsomes (16, 17). The K_i determined for FPP (Table 1) was lower than the K_m values of approximately 5–24 μM for this compound in squalene synthesis or condensation reactions catalyzed by *cis*-prenyltransferase (6, 18). The K_i determined for FPP (Table 1) using recombinant human MKase approximated the FPP K_m value of approximately 0.1 μM for protein farnesyltransferase from rat brain cytosol (18). These data suggest that intracellular GPP and FPP concentrations are sufficient for feedback repression of mammalian MKase activity at the protein level. We found that the C_{20} compound, geranylgeranyl-diphosphate, was the most potent inhibitor, with a K_i of approximately 59 nM (Table 1). A summary of K_i determinations using FPP and GGPP is depicted in Fig. 3. One explanation for the significantly lower K_i values observed in our studies is the probability that our enzyme preparations, obtained through expression of cDNAs in bacteria with subsequent affinity purification, are more pure than enzyme preparations used in earlier studies (5, 6).

IPP and DMAPP competitively inhibited recombinant human MKase with respect to ATP, yielding K_i values of 16–20 μM (Table 1). Dorsey and Porter (5) found these compounds to be weak inhibitors of porcine liver MKase, with 0.5 mM IPP and 0.59 mM DMAPP inhibiting 27% and 57%, respectively, of porcine MKase activity. The K_i values determined in the present study were an order of magnitude lower than these values. The K_m for IPP in the rat FPP synthase reaction is approximately 2 μM (16), while the K_m for IPP in the *cis*-prenyltransferase reaction in embryonic rat brain cells is 33 μM (19). These values argue against a role for IPP and DMAPP as physiologic regulators of MKase in vivo. Of interest, several of the compounds active against MKase had no inhibitory effect against PMKase or MDDase when tested (Table 1).

Our results serve to expand the spectrum of physiologic inhibitors that potentially regulate mammalian MKase activity. It appears that the optimal chain length for inhibition of MKase is C_{15}–C_{20}, and the diphosphate form of the intermediate appears important. This is exemplified by the difference in inhibition constants for FPP versus farnesol alone (Table 1). It is of interest that

farnesol can inhibit MKase. This inhibition suggests that the ATP binding motif in MKase must be very hydrophobic, consistent with the overall high index of hydrophobicity of this protein (3). Although the C_{10} inhibitor, GPP, was still a strong competitive inhibitor, once the chain length was decreased to C_5 (as in the case of DMAPP and IPP), the K_i for competitive inhibition of MKase increased by a factor of approximately 10^3 (Table 1). Consistent with these observations, the C_5 compound mevalonate 5-diphosphate, which is substantially more polar than IPP and DMAPP, did not inhibit MKase activity.

An unexpected finding in our work, and another argument supporting the hydrophobic nature of the MKase ATP binding pocket, was the ability of DP to competitively inhibit human MKase (Table 1). The inability of dolichol alone, at concentrations five times those used to inhibit MKase activity by DP, argued against the possibility that inhibition of MKase by DP was due solely to a detergent effect of dolichol alone. Although ER-associated *cis*-prenyltransferase is the rate-limiting step in DP synthesis, there is evidence that decreasing the mevalonate pool can lead to reduction in DP concentrations. Studies using L6 myoblasts have indicated that depletion of the intracellular mevalonate pool through incubation of cells with lovastatin can result in decreased DP biosynthesis (20). Ericsson and coworkers (21) estimate that 50% of hepatic dolichol biosynthesis occurs within the peroxisome, and Biardi et al. (22) and Biardi and Krisans (23) have demonstrated that MKase is almost exclusively localized in this organelle. Other groups have suggested that dolichol phosphate is constantly produced by the cell and very slowly (if at all) metabolized (24, 25). These data raise the possibility that a reduction in intracellular levels of FPP, via reduction in intracellular mevalonate pools, could lead to decreased DP synthesis, which might argue in favor of a physiologic role for DP in the regulation of MKase activity in vivo. To further assess this possibility, we studied the effect of DP on MKase in crude bacterial extracts and found that MKase activity could be significantly inhibited. Further analysis of the physiologic significance of DP inhibition of MKase activity in vivo will be needed to establish a role of DP in control of MKase activity.

Our results indicate that of the three ATP-dependent enzymes involved in the early steps of mevalonate catabolism, MKase is a specific site of feedback regulation. Comparison of the amino acid sequences of MKase, PMKase, and MDDase may provide insights. In the MKase coding sequence, there are two highly conserved regions that have been implicated in ATP binding in other kinases (14). One of these conserved elements contains the sequence G-X-G-X-X-G-X_{15-21}-A-X-K (where X is any amino acid), occurring near the end of the MKase protein (amino acid residues 333–357, single letter amino acid sequence GAGGGGCGITLLKP GLEQPEVEATK). Conversely, a consensus ATP binding site predicted by the PROSITE protein program is [LIVM]-P-X-[GSTA]-X(0,1)-G-L-[GS]-S-S-[GSA]-[GSTAC]. In the PROSITE program, any of the bracketed amino acids may occupy that position in amino acid sequence (single letter amino acid code), and X represents any amino acid. Where X(0,1) occurs, this indicates that none or one amino acid may occur in this position. A PROSITE consensus sequence occurs in MKase at amino acid residues 137–148 of the polypeptide as LPPGAGLGSSAA (14). The deduced amino acid sequences of PMKase has none of these elements, while MDDase contains the sequence FPTAAGLASSAA motif which is highly homologous to the PROSITE prediction (11, 26). In terms of MKase, which of these elements is active in ATP binding awaits site-directed mutation analysis, but it remains likely that the structure of the ATP-binding pocket of MKase, or perhaps the overall hydrophobicity of the enzyme, enables selective inhibition by isoprenes in the early pathway of mevalonate disposition, which may have physiologic significance in vivo.

The pathway of cholesterol and nonsterol isoprene biosynthesis is subject to multivalent transcriptional and post-transcriptional regulation, primarily at the level of HMG-CoA reductase (1). Given the importance of the products derived from these pathways, this is not surprising. The reaction catalyzed by HMG-CoA reductase is believed to be both the primary point of pathway regulation and the rate-limiting step. However, it is becoming increasingly clear that MKase, the enzyme responsible for catabolism of the reductase reaction product, is also subject to multilevel regulation, both at the transcriptional and post-translational level. Similarly, the reaction catalyzed by PMKase appears to respond to sterol levels through transcriptional controls (26). Additional studies of the genes encoding the three ATP-dependent enzymes involved in mevalonate disposition (Fig. 1), and the enzyme products themselves, will no doubt reveal additional insights into the regulation of the early region of this important biosynthetic pathway.

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