Activity and Autophosphorylation of LAMMER Protein Kinases*

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EXPERIMENTAL PROCEDURES

Expression and Purification of LAMMER Kinases—Expression and purification of fusion proteins utilized the pMAL-C2 vector of New England Biolabs and were performed as recommended. DOA, a StyI fragment of cDNA clone, pCD1 + 2, containing the DOA catalytic domain (7), was modified with EcoRI linkers and inserted into pMAL-C2. SK-G1, a Bar-Bi-BamHI fragment containing the catalytic domain of pSK-G1, generously provided by Steven Hanks, was modified with BamHI linkers and inserted into pMAL-C2. KNS1, a Sept-BamHI fragment from p217, containing the KNS1 catalytic domain, kindly provided by Michael Snyder and Ramesh Padmanabha (18), was modified with BamHI linkers and inserted into pMAL-C2. Expression and purification of fusion proteins was confirmed by probing immunoblots of crude and affinity-purified proteins with antibodies directed against the maltose binding domain. In all cases, a protein of the expected molecular weight was observed. Affinity-purified fusion proteins obtained

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Protein kinases are generally differentiated according to the specificity with which they phosphorylate substrates, with activity toward serine/threonine or tyrosine residues (1, 2). Recently, some kinases capable of phosphorylating all three amino acid residues have been identified (reviewed in Refs. 3 and 4). Such “dual specificity” kinases would be classified as serine/threonine-specific based on their amino acid sequences. Among these is murine STY or Clk (5, 6), whose cDNAs were isolated because their products autophosphorylated on tyrosine residues. Data base searches with the sequence of cDNAs from the Doa locus of Drosophila revealed murine Clk and other closely related homologues in eukaryotes ranging from yeast to humans (7). Additional homologues have subsequently been described in green plants (8) (also accession number Z69239). We have dubbed these the LAMMER protein kinases, based upon the existence of this motif, or conserved variations upon it, in all members (7).

The LAMMER kinases are nearly identical in size, spacing, and placement of their kinase catalytic domains and also show extremely high amino acid identity in domains essential for phosphotransfer to the peptide substrate and in substrate recognition (11). By analogy with the structure of two crystallized protein kinases (12, 13), the LAMMER motif lies in an α-helix below the substrate-binding cleft, potentially allowing it to make contact with substrates, and suggesting that these molecules are also highly conserved.

Much of what is known of LAMMER kinase function derives from analysis of the Drosophila locus Doa, whose mutants were isolated during screens for loci with transcriptional regulatory effects (14, 15). Doa is an essential gene; its mutations alter the mutagenic activity and transcription rate of the copia retrotransposon and reveal defects in embryonic segmentation and nervous system development, degeneration of retinal photoreceptors, and small imaginal discs (7). Recently, murine Clk was described as phosphorylating and altering intranuclear localization of SR splicing factors, suggesting that these proteins are among the in vivo substrates of LAMMER kinases (16).

Murine Clk autophosphorylates on serine, threonine and tyrosine residues in vivo, suggesting that there may be a biological function for dual specificity (17). To begin to address this issue further, we have examined whether other LAMMER putative protein kinases autophosphorylate in vitro, as well as whether they too possess dual specificity. We report here that DOA and the LAMMER kinases SK-G1 of humans (recently reidentified as Clk2 (9)) and KNS1 of Saccharomyces cerevisiae possess protein kinase activity and autophosphorylate with dual specificity in vitro, as murine Clk/STY, suggesting that the entire family possesses this activity. Although the LAMMER kinases are closely related to the mitogen-activated protein kinase family, they possess different substrate specificity in vivo, based on phosphorylation of peptide and protein substrates and sequencing of a phosphorylation site in a common substrate.
were: DOA, 85 kDa, with some apparent degradation products; SK-G1, 83 kDa; KNS1, 106 kDa, 90 kDa, and 78 kDa, the latter two and a smear at about 40 kDa thought to be due to degradation.

Site-directed Mutagenesis—Site-directed mutagenesis altering Lys-199 (AAG) to Arg (AGG) in subdomain II of DOA utilized the Promega Altered Sites In Vitro Mutagenesis System, as per instructions. A 0.3-kilobase BamHI fragment excised from the pMAL-DOA expression vector was inserted into the pALTER-1 vector. The mutagenic oligonucleotides (5'-TAC TGC ATG GCT TTA AGG ATT ATT AAG AAC GTG-3') replaced the native sequence, as confirmed by DNA sequencing.

SDS-Gel Electrophoresis, Protein Transfers, and Immunoblots—SDS-gel electrophoresis, protein transfers, and immunoblots were performed as described (19), using a 10% separating gel. Proteins were transferred to nitrocellulose by electrophoretic blotting for immunological detection and renaturation kinase assays. Immunological detection utilized the Amersham ECL chemiluminescence system.

Autophosphorylation, Kinase Assays, and Phosphatase Treatment—Filter denaturation/renaturation kinase assays were performed as described (20, 21), using 1 ml of bacterial lysates or 5–10 μg of electrophoresed purified fusion proteins, which were transferred to nitrocellulose for renaturation. Purified KNS1 fusion protein was autophosphorylated by incubation in the same buffer.

Kinase activity toward exogenous substrates was tested in 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂, 20 mg/ml bovine serum albumin, 0.5 mg/ml synthetic peptides or protein substrates, 1 μl of 500 μM γ⁻³²P[ATP], and 1–50 μg/ml purified DOA fusion protein. Reactions were incubated for 20 min at room temperature, stopped by spotting 25-μl aliquots onto Whatman P-81 phosphocellulose paper, and washed in 75 mM phosphoric acid 5 times, 5 min each, dried, and counted.

Specific activity determinations of the kinases toward MBP were performed in 25 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, and 100 μM ATP, incubated 10 min at 30 °C. A unit of LAMMER kinase activity was arbitrarily defined as the amount of kinase required to catalyze the transfer of 1 pmol of phosphate to MBP in 1 min at 30 °C, in a 100-μl reaction volume. Reactivated recombinant murine ERK2 MAP kinase was purchased from New England Biolabs.

Dephosphorylation was accomplished by incubating cell lysates for 30 min at 37 °C with calf intestinal phosphatase in 50 mM Tris-HCl, pH 8.0, 0.1 mg/ml phosphatase (DAG 7.4, as a competitive inhibitor).

Phosphoamino Acid Analysis—Identification of phosphoamino acids was accomplished by incubating cell lysates for 30 min at 37 °C with calf intestinal phosphatase in 50 mM Tris-HCl, pH 8.0, 0.1 mg/ml phosphatase (DAG 7.4, as a competitive inhibitor).

Results

LAMMER Kinase Homologues Possess Protein Kinase Activity—To determine whether LAMMER family members possess kinase activity and whether they autophosphorylate with dual specificity, analogously to murine Clk, we characterized the activity of the gene products encoded by Doa of Drosophila, human SK-G1 (Clk2), and KNS1 of S. cerevisiae. These family members were selected based on their wide evolutionary divergence. Our hypothesis was that if these three LAMMER kinases possess dual specificity, that all family members are likely to.

3 R. G. Cook, personal communication.

The catalytic domains of the selected family members were expressed and purified as maltose-binding fusion proteins. Affinity purified proteins of the predicted size were obtained in each case (Fig. 1), although both DOA and KNS1 also yielded several lower molecular weight proteins, presumably due to degradation or lability.

Both crude extracts and affinity-purified proteins were tested for kinase activity, by subjecting extracts of electrophoresed proteins to a filter-based autophosphorylation assay (21), identical to that used for murine Clk (6). In the case of the KNS1 fusion, this assay demonstrated only a very weak signal, and so purified protein was assayed directly in solution, followed by separation on an SDS gel, and autoradiography. Positive and negative controls were provided by crude extracts in which a TrpE-murine Clk fusion and the pMAL vector had been expressed, respectively.

Crude extracts and purified fusion proteins of all three putative kinases possess autophosphorylation activity at the molecular weight of the purified fusion protein and, in the case of KNS1, a subset of the degradation products (Fig. 1). No activity was observed in extracts expressing the vector alone. Further verification that the kinase activity originated only from the fusion proteins was provided by cleaving the purified DOA fusion protein into its maltose-binding and DOA moieties with factor Xa. Digested reaction products subjected to kinase renaturation assays revealed two labeled bands corresponding to the molecular weights of the uncleaved protein and the excised DOA catalytic domain (Fig. 1).

Lysine 199 of DOA corresponds to an invariant residue in...
kinase catalytic domain II of all protein kinases, and alteration of this residue to any other drastically reduces kinase catalytic activity (1, 2). As final confirmation that the activities observed in the LAMMER kinase preparations were due solely to the cloned kinases, Lys-199 of DOA was altered by site-directed mutagenesis to an Arg. The (K199R) mutant protein possessed less than 2% of the activity in crude extracts, compared with equivalent amounts of crude extracts in which the wild type protein was expressed (Fig. 1), providing conclusive proof that bacterially expressed DOA and the other two LAMMER putative kinases possess intrinsic kinase activity.

It was noted that the mutant DOA fusion protein (K199R) migrated very slightly faster than the wild type fusion protein on SDS gels. To determine whether this differential migration was due to autophosphorylation of the wild-type protein in E. coli, purified wild type DOA fusion protein was treated with calf intestinal phosphatase prior to electrophoresis. Phosphatase treatment increased the migration of wild type DOA fusion protein to that of the catalytically inactive fusion (not shown). Faster migration of the treated wild-type protein did not occur when the phosphatase was boiled prior to treatment or phosphatase treatment took place in the presence of sodium phosphate, a nonspecific phosphatase inhibitor. We conclude that the DOA fusion protein autophosphorylates in E. coli, consistent with the isolation of murine Clk as a tyrosine-phosphorylated protein from expression cDNA libraries (5, 6).

LAMMER Protein Kinases from Drosophila, Humans, and S. cerevisiae Autophosphorylate with Dual Specificity—To determine the autophosphorylation specificity of the three kinases, phosphoamino acid analysis was performed on the purified fusion proteins. These results conclusively demonstrate that all three autophosphorylate with dual specificity, albeit with differing efficiency toward tyrosine residues (Fig. 2).

In the case of DOA, the phosphoserine signal comprised 96.8% of the total activity, phosphothreonine 2.5%, and phosphotyrosine was barely detectable at 0.7% (Fig. 2 and Table I). To verify the phosphotyrosine signal, proteins on the PVDF membranes were base-hydrolyzed prior to acid hydrolysis, to hydrolyze most of the phosphoserine and phosphothreonine (22). Since phosphotyrosine is not labile in alkali, its signal is enhanced under these conditions. Phosphotyrosine was also detected by grossly overloading the thin layer chromatography plates with sample prepared by standard acid hydrolysis (Fig. 2). All three methods demonstrate that DOA autophosphorylates in vitro with dual specificity, albeit weakly on tyrosine.

The autophosphorylation specificity of SK-G1 also shows that phosphoserine is the major phosphorylated amino acid, although phosphothreonine and phosphotyrosine are easily detected (Fig. 2 and Table I). Purified pMAL-KNS fusion proteins were also tested. As for SK-G1 and DOA, a prominently labeled 65-kDa KNS1 was heavily phosphorylated on phosphoserine, with phosphothreonine and phosphotyrosine also detected (Fig. 2 and Table I). Thus, all LAMMER protein kinase family members tested autophosphorylate with dual specificity. KNS1 is among the most diverged in amino acid sequence of LAMMER family members, and so these results suggest that they all may possess this property.

LAMMER Kinases Phosphorylate Myelin Basic Protein in Vitro—To identify in vitro substrates for DOA kinase, 13 synthetic peptides, myelin basic protein, and histone H1 were tested (Table II). The two intact proteins were chosen because they serve as in vitro substrates for many protein kinases, including MAP kinase and p34cdc2 close phylogenetic relatives of the LAMMER kinases. The synthetic peptides were chosen essentially at random. However, MAPK and p34cdc2 substrates were of particular interest due to the close relationship between these families and the LAMMER kinases. Of the extended protein kinase family, the LAMMER kinases are most closely related to the MAP kinases. For example, the catalytic domain of murine Clk shares 55% amino acid identity with the MAP kinase FUS3 of S. cerevisiae (6), sharing high conservation in residues and domains important in phosphotransfer and substrate recognition.

Of the proteins and peptides tested, DOA efficiently phosphorylates only MBP, in vitro, stimulating phosphate incorporation over 80-fold above background. MBP is also an efficient in vitro substrate for MAPK. However, although both MAPK and DOA phosphorylate intact MBP, three peptides phosphorylated by MAPK (the sequences from MBP, tyrosine hydroxylase, and the epidermal growth factor receptor phosphorylated by MAPK) were not phosphorylated by DOA, suggesting that the phosphorylation site of DOA is different from that of MAPK.
To compare the relative activities of the LAMMER kinases with MAP kinase toward their common substrate, MBP, we performed in vitro phosphorylation reactions with DOA, KNS1, SK-G1 (Clk2), and the MAP kinase ERK2, using [32P]ATP (Fig. 3). Following correction for the presence of the maltose-binding protein fusion domain in the LAMMER kinases, the specific activities of the kinases toward MBP are as follows: DOA, 3.0 units/μg; SK-G1, 11.5 units/μg; KNS1, 0.16 units/μg. These figures compare to the approximate 500 units/μg of the recombinant, commercially supplied MAP kinase. It might be noted that these figures are quantitative estimates of LAMMER kinase activity in vitro and may not reflect the actual activity of the kinases in vivo, since they are susceptible to influences by the structure of the fusion protein, among other things.

To identify the LAMMER kinase phosphorylation sites on MBP, labeled protein was isolated on a SDS gel, tryptic digests were performed on the excised gel slice, and peptides were separated by HPLC. Two peptides eluting at virtually identical points from the column were radiolabeled by DOA, SK-G1, and KNS1. N-terminal sequencing of these peptides revealed that they correspond to residues 162–168 (SGSPMAR) or 162–169 (SGSPMARR) of bovine myelin basic protein, the second serine in each case being the labeled residue. As hypothesized, this site is different from the major phosphorylation site in bovine MBP for MAP kinases, which has been previously defined as 9NIVTPRTPE26 (26, 27).

Murine Clk phosphorylates the synthetic peptide poly(Glu, Tyr), which was interpreted as suggesting that it may possess tyrosine specificity on exogenous substrates (6). We therefore determined whether DOA also would phosphorylate poly(Glu, Tyr); no significant differences from controls (no substrate) were observed. Thus, DOA does not efficiently phosphorylate poly(Glu, Tyr), in contrast to its murine relative, Clk. This difference may be attributable to any of a number of factors, including the possibility that the general tyrosine kinase activity of the DOA fusion construct is significantly less than Clk’s, as suggested in the phosphoamino acid analyses reported above.

**DISCUSSION**

Although no specific motifs have yet been identified that confer dual specificity on a protein kinase, the high sequence identity among the LAMMER family and the dual specificity of murine Clk suggested that additional family members might autophosphorylate with dual specificity. It is therefore not surprising that the three LAMMER homologues DOA, SK-G1, and KNS1 not only encode active protein kinases, but autophosphorylate with dual specificity. These results suggest that all other LAMMER family members possess this property, since KNS1, the S. cerevisiae family member, is among the most diverged in the family.

Attempts to identify DOA substrates in vitro among several proteins and synthetic peptides showed that MBP was efficiently phosphorylated in vitro, although no others were found. N-terminal peptide sequencing revealed that Ser-164 of bovine MBP is phosphorylated by DOA, SK-G1, and KNS1, supporting the hypothesis that the LAMMER kinases will have very similar substrate specificities. Given the structural relationships between the LAMMER family and the MAP kinases and Cdk’s, it is, however, not surprising that the major phosphorylation site of DOA, SK-G1, and KNS1 in MBP possesses a Pro residue at the +1 position, since the consensus phosphorylation sites for the latter two families also show similar specificity. Thus, although closely related, the LAMMER and MAP kinases apparently possess different substrate specificities, based on the fact that the major site of MBP phosphorylation by MAP kinases is different from the one phosphorylated by DOA, SK-G1, and KNS1, as well as on the failure of DOA to phosphorylate any of the MAP kinase peptide substrates tested. Definition of a consensus LAMMER kinase phosphorylation site awaits further experimentation.

In contrast to murine Clk, DOA did not phosphorylate poly(Glu,Tyr). This result may be explained by the very low efficiency with which DOA autophosphorylates on tyrosine residues, in contrast to murine Clk. Alternatively, neither result may be indicative of the true activity of these kinases in vivo, since these assays were performed in vitro with bacterially...
expressed fusion proteins.

The significance of the dual specificity autophosphorylation of LAMMER kinases remains to be determined. It is known that the murine homologue Clk autophosphorylates on tyrosine when expressed in 3T3 cells (17). Autophosphorylation with dual specificity may not be biologically relevant in vivo and may not accurately reflect substrate specificity (3). However, as in the case of many tyrosine kinases, autophosphorylation on one or more tyrosine residues might allow docking of either regulatory or substrate molecules containing SH2-binding sites (for reviews see Refs. 28 and 29). If the dual specificity of the LAMMER kinases is biologically significant, even extremely low stoichiometries of tyrosine autophosphorylation may be important in the creation of such SH2 domain docking sites. Only the identification of additional molecules interacting with the LAMMER kinases will reveal the significance or irrelevance of autophosphorylation with dual specificity.

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