Characterization of a Novel Serine/Threonine Kinase Associated with Nuclear Bodies*

(Received for publication, July 26, 1999, and in revised form, December 7, 1999)

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A novel protein kinase, Mx-interacting protein kinase (PKM), has been identified in a yeast two-hybrid screen for interaction partners of human MxA, an interferon-induced GTPase with antiviral activity against several RNA viruses. A highly conserved protein kinase domain is present in the N-terminal moiety of PKM, whereas an Mx interaction domain overlaps with C-terminal PEST sequences. PKM has a molecular weight of about 127,000 and exhibits high sequence homology to members of a recently described family of homeodomain-interacting protein kinases. Recombinant PKM has serine/threonine kinase activity that is abolished by a single amino acid substitution in the ATP binding domain (K221W). PKM catalyzes autophosphorylation and phosphorylation of various cellular and viral proteins. PKM is expressed constitutively and colocalizes with the interferon-inducible Sp100 protein and murine Mx1 in discrete nuclear structures known as nuclear bodies.

Nuclear bodies (NBs) are nuclear structures of undefined function (1) that are also known as nuclear dots (2), nuclear domain 10 (3), or promyelocytic leukemia protein (PML) oncogenic domains (4). NBs consist of several components, including interferon (IFN)-induced proteins such as PML and the primary biliary cirrhosis autoantigen Sp100 (5, 6). NB components seem to be involved in gene regulation, control of cell growth, and apoptosis. Infection of cells by various viruses influences the composition and integrity of NBs, suggesting a function of NBs in early viral infection and antiviral response (2, 9). For example, the immediate early gene product ICP0 of herpes simplex virus-type I associates with NBs in the early phase of infection and leads to a complete loss of NB-specific staining (7). In contrast, infection with influenza virus (FLUAV) increases the number and staining intensity of NBs in much the same way as does treatment with type I IFN (5).

Moreover, PML contributes to the antiviral state induced in IFN-treated cells by having selective antiviral activity against vesicular stomatitis virus (VSV) and FLUAV but not encephalomyocarditis virus (8). Furthermore, the IFN-induced murine Mx1 protein forms nuclear dots (9) that have been found to be partially associated with NBs (10). Mx proteins are large guanine triphosphatases (GTPases) that are tightly regulated by type I IFNs (11) and display antiviral activity against a variety of RNA viruses (12, 13). The antiviral mechanism of Mx proteins is poorly understood, and it has been proposed that they require the help of constitutive host cell factors for their function and antiviral specificity (14, 15). To identify cellular factors possibly involved in antiviral or other functions of Mx proteins, we performed a yeast two-hybrid screen of a cDNA library, using MxA as a bait. Here we report the identification and characterization of a 127-kDa protein kinase that interacts with Mx protein family members and hence is termed PKM for Mx-interacting protein kinase.

MATERIALS AND METHODS

Yeast Two-hybrid Constructs and Screening—A two-hybrid library, representing mRNAs expressed in baby hamster kidney (BHK-21) cells infected with Thogoto virus (THOV strain Sia126 (17)), was constructed in the HybridZAP vector (Stratagene). Poly(A)*-selected RNA was used to synthesize the cDNA library following the manufacturer's protocol (Stratagene). The resulting library pAD-BHK/THOV consisted of 2.4 × 10^6 independent clones with an average size of 1300 base pairs. The bait plasmid, pBD-MxA, was constructed by cloning nucleotides 236–2243 of human MxA (18) into pBD-GAL4 (Stratagene). The two-hybrid library screen was performed according to the manufacturer's protocol (Stratagene). Briefly, the Saccharomyces cerevisiae yeast strain YRG-2 was sequentially transformed with the bait plasmid pBD-MxA and the pAD-BHK/THOV library DNA using the lithium acetate method. MxA-interacting proteins were identified by growth on SD minimal medium lacking tryptophan, leucine, and histidine. Positive clones were verified by assessing their interaction with pBD-MxA versus two control baits, pBD-GAL4 and pBD-NP, the latter encoding a THOV nucleoprotein-GAL4 DNA binding domain hybrid.

Molecular Cloning of PKM and Plasmid Constructs—The 5′-end of clone 512 was determined by 5′-rapid amplification of cDNA ends (5′-RACE; Life Technologies, Inc.) with RNA of BHK-21 cells and two internal primers (nucleotides 959–930 and 701–672 of the later PKM cDNA). The 5′-extended open reading frame (ORF) of clone 512 was amplified from BHK-21 RNA by reverse transcriptase-polymerase chain reaction and was combined with clone 216 using a unique ApaLI restriction site within the overlapping region. The resulting full-length ORF was cloned into the eukaryotic expression vector pcDNA3 (19) yielding the plasmid pCM-PKM. For expression of glutathione S-transferase (GST) fusion proteins, the ORF of clone 512 was inserted into the prokaryotic expression vector pGEX-4T-1 (Amersham Pharmacia Biotech) yielding pGEX-PKM (148–925). Mutant PKM (K221W) was generated by replacing the codon for lysine 221 with the codon for tryptophan utilizing QuickChange site-directed mutagenesis (Stratagene). The introduced mutations were confirmed by sequencing.

Cells—Embryonic fibroblast cells of the mouse strains A2G and BALB.A2G-Mx (20) and the cell lines T98G (21), BHK-21, and COS-1 were grown in Dulbecco's modified essential medium containing 10% fetal calf serum.

* This work was supported by Grant HA 1582 from the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF144573.

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The abbreviations used are: NB, nuclear bodies; IFN, interferon; PKM, Mx-interacting protein kinase; BHK, baby hamster kidney; THOV, Thogoto virus; RACE, rapid amplification of cDNA ends; ORF, open reading frame; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; HIPK, homeomain-interacting protein kinase; GTP-γS, guanosine 5′-3′-O-thiotriphosphate; NP, nucleoprotein; PML, promyelocytic leukemia protein; FLUAV, influenza A virus; VSV, vesicular stomatitis virus.
**Mx-interacting Protein Kinase PKM**

**FIG. 1.** Domain structure of PKM and predicted amino acid sequence. A, schematic representation of PKM and the two fragments (512 and 216) isolated by yeast two-hybrid interaction cloning. The full-length PKM cDNA is composed of the overlapping clones 512 and 216. Clone 512 was elongated by 5'-RACE (white box). A conserved Ser/Thr-kinase domain (black boxes (33)) and PEST sequences (gray boxes (34)) are indicated. The overlapping region of clones 512 and 216 contains the Mx interaction domain (black bar). Numbers indicate amino acid positions. B, alignment of amino acid sequences of hamster PKM (AF144573) and mouse HIPK2 (AF077659) (16). Amino acid differences are shaded, and gaps are indicated by dashes. The ATP binding region (amino acids 192–248) and the putative Mx interaction domain of PKM (amino acids 792–925) are underlined. The conserved lysine (K) residue at position 221 used to generate the kinase-defective (K221W) mutation is indicated by an asterisk.

**RESULTS AND DISCUSSION**

In a yeast two-hybrid search for cellular and viral interaction partners of human MxA, two overlapping cDNA clones were isolated from a cDNA library of THOV-infected BHK-21 cells. They encode parts of the ORF of a putative serine/threonine kinase. To obtain the full-length ORF, the cDNA was extended by 5'-RACE (32). Within this extension, a translational start codon was present at position 174–176. The resulting full-length cDNA had a length of 3909 base pairs and coded for a protein of 127 kDa. Sequence analyses located a conserved protein kinase domain (33) in the N-terminal half of the protein as well as PEST sequences (34) in the C terminus (Fig. 1A). The full-length protein, the two original fragments, and their overlapping region showed clear interactions with human MxA and mouse Mx1 protein in the two-hybrid assay, indicating the presence of a putative Mx interaction domain (Fig. 1A). Therefore, the kinase was named PKM, for protein kinase interacting with Mx proteins. However, pull-down assays or co-immunoprecipitations failed to reveal a direct biochemical interaction between PKM and MxA (data not shown), indicating that stable complexes are not readily formed under these conditions. It has been notoriously difficult to demonstrate direct binding of MxA with putative cellular or viral interaction partners (23, 35), and it remains to be seen whether a weak or transient interaction of Mx proteins with PKM will be demonstrable in the future, using more sensitive technologies.
having an insertion of 27 amino acids (amino acids 588–614) (Fig. 1B). Interestingly, the putative Mx interaction domain has 40 amino acids in common with the homeoprotein interaction domain (Fig. 1B). Sequence similarities extending beyond the catalytic domain were also found between PKM and the rat androgen receptor-interacting protein kinase (48.8%; (36)) as well as the human YAK1-related protein kinase PKY (46.6% (37)). In addition, the catalytic domain of PKM showed strong similarity to the equivalent domain of the YAK1-related rat kinase DYRK, which is a dual specificity protein kinase catalyzing phosphorylation on both serine/threonine and tyrosine residues (38–40). The sequence data indicate that PKM is a new member of the growing family of homedomain-interacting protein kinases.

Gene expression of PKM was investigated in BHK-21 cells. Northern blot analysis of total RNA detected three transcripts of 15.0, 6.3, and 4.3 kilobases (Fig. 2). All three bands represented PKM-specific transcripts that were detectable with two independent probes (Fig. 2, lanes 1 and 2). They could not be detected in RNase-treated samples (data not shown), indicating that the 15.0-kilobase signal represented an RNA transcript. When poly(A)^+ selected RNA was used instead of total RNA, a single mRNA species corresponding to the 4.3-kilobase transcript was detected (Fig. 2, lane 3). Therefore, we conclude that PKM is expressed in BHK-21 cells and that the 4.3-kilobase band represents the mature polyadenylated mRNA. The larger transcripts most likely represent incompletely processed transcripts lacking a poly(A) tail. The expression level of PKM was not altered by infection with two different orthomyxoviruses, namely FLUAV and THOV, or by treatment with 2000 units/ml IFN-a/b/D (30) for 16 h (not shown).

To demonstrate that PKM has protein kinase activity, a recombinant GST fusion protein was expressed in E. coli and purified by affinity adsorption on glutathione-agarose beads. Because the full-length 127-kDa kinase could not be obtained in substantial amounts, a PKM fragment (amino acids 148–925) containing the entire kinase domain but lacking N- and C-terminal sequences was expressed as a GST fusion protein. The purified GST-PKM-(148–925) protein showed the expected apparent molecular weight of 120,000 as revealed by SDS-PAGE (Fig. 3A, lane 1). A second band with an apparent molecular weight of 85,000 was observed, which most likely represented a major degradation product. GST-PKM-(148–925) catalyzed autophosphorylation in an in vitro kinase assay, demonstrating kinase activity of the fusion protein (Fig. 3A, lane 3). The 85-kDa degradation product was also phosphorylated. To exclude the possibility that the observed ^32P incorporation was caused by a contaminating protein kinase, a kinase-defective mutant was generated. Within the ATP binding site of PKM, lysine 221 was changed to tryptophan (K221W) corresponding to a mutation in Raf-1 that abolished its kinase activity (41). As expected, the mutant protein GST-PKM-(148–925, K221W) lacked kinase activity (Fig. 3A, lanes 2 and 4). Compared with GST-PKM-(148–925), the mutant protein exhibited a slightly higher mobility in SDS-PAGE (Fig. 3A, lane 2) most likely reflecting the absence of phosphate groups. These findings demonstrate that PKM has intrinsic kinase activity.

To further characterize the activity of PKM, in vitro kinase assays were performed with exogenous substrates. Fig. 3B shows that E. coli-expressed GST-PKM-(148–925) catalyzed ^32P incorporation into classical substrates as histone, casein, and myelin basic protein but failed to phosphorylate bovine serum albumin. Purified MxA protein produced in E. coli was not phosphorylated by PKM, also not in the presence of the nonhydrolyzable nucleotide analog GTP-$\gamma$S known to stabilize the activated conformation of MxA (28). In contrast, the E. coli-produced nucleoprotein (NP) of THOV was phosphorylated by GST-PKM-(148–925), whereas the same protein purified from virus particles was not (Fig. 3B). Because NPs of orthomyxoviruses are phosphoproteins (42), it is conceivable that the relevant residues were already equipped with a phosphate group preventing further phosphorylation by PKM. The cellular protein kinases that mediate phosphorylation of THOV NP in infected cells are presently not known. It remains to be seen whether PKM or other HIPK family members are involved.

To investigate the kinase specificity of full-length PKM, a cDNA coding for FLAG-tagged full-length PKM was expressed in COS-1 cells using the vaccinia T7 polymerase system (22). Cell lysates were prepared, and PKM was immunoprecipitated with an anti-FLAG antibody. The immobilized PKM showed autophosphorylation activity and accepted cellular as well as viral proteins as substrates (Fig. 3C). Thus, PKM phosphorylated the E. coli-produced phosphoprotein of VSV and the NPs of FLUAV (FLUAV NP) and THOV (THOV NP). A full-length kinase-inactive mutant PKM (K221W) was found to exhibit no

### Table I

| Kinase | haPKM mouse | mHIPK2 mouse | huPKY human | rANPK rat | rDYRK rat | YAK1 yeast |
|-------|-------------|--------------|-------------|-----------|-----------|------------|
| % nucleotide identity | 58.5 | 58.4 | 24.6 | 18.2 | 18.7 | 20.0 |
| % amino acid identity | 48.8 | 48.4 | 19.9 | 19.7 | 20.3 | 19.9 |

**Fig. 2. PKM transcripts.** Total RNA (15 µg/lane) or poly(A)^+ selected RNA (1 µg) was isolated from BHK-21 cells and analyzed by Northern blotting (see “Materials and Methods”). The blot was probed either with a cDNA fragment of PKM corresponding to nucleotides 1–701 (lane 1) or with a central fragment corresponding to nucleotides 2292–2953 (lanes 2 and 3). Positions of RNA standards are indicated on the left. nt, nucleotide.
kinase activity (data not shown). These data demonstrate that full-length PKM has kinase activity and phosphorylates a similar set of proteins as the truncated GST-PKM-(148–925).

The kinase domain of PKM showed high sequence homology to conserved kinase domain of DYRK (Table I). This dual specificity protein kinase is able to phosphorylate both serine/threonine and tyrosine residues (38). These kinases share the domains common to all serine/threonine kinases but have otherwise no known motifs predicting dual specificity (43). We therefore checked the specificity of PKM by phosphoamino acid analyses of PKM substrates. Fig. 3D shows that PKM catalyzed its own phosphorylation on serine and threonine residues. THOV NP was phosphorylated preferentially on serines, whereas casein was phosphorylated on threonines. No evidence for tyrosin phosphorylation was found, indicating that PKM is a true serine/threonine kinase, rather than a dual-specific kinase.

Finally, we investigated the subcellular localization of PKM. FLAG-tagged PKM was transiently expressed in mouse primary embryo cells (Fig. 4A) as well as in human T98G, COS-1, and Swiss 3T3 cells (data not shown). It localized to distinct spots within the nucleus, demonstrating that PKM is a nuclear protein kinase. Because the dot-like appearance of nuclear PKM much resembled the intranuclear distribution of PML, we investigated whether PKM was found in NBs of IFN-treated and PKM-transfected cells, using Sp100 as a marker protein (2). Fig. 4A shows that PKM indeed colocalized with Sp100 in distinct nuclear dots, suggesting that PKM belongs to the NB-associated cellular proteins. The PKM-related kinase HIPK2 was also detected in nuclear speckles (16, 44), suggesting that HIPKs may be a group of kinases that preferentially associate with NBs. Recent data by Kim et al. (44) demonstrate that HIPK2 is modified by the small ubiquitin-like protein SUMO-1. It has previously been shown that posttranslational modification by SUMO-1 directs a subset of nuclear proteins to the NBs (2, 45). The organized structure of NBs is disturbed in a number of pathological processes, indicating that the integrity of NBs is important for distinct cellular functions (1, 2, 3). NBs reportedly play a role in growth control, cell transformation, cellular stress responses, and IFN action. The fact that IFNs up-regulate some NB-associated proteins and the recent finding that viruses have evolved strategies to disrupt or reorganize NBs suggest that NBs may have a significant role in virus-host interactions. Interestingly, Mx1 was described to be localized in or partially associated to NBs (10). Moreover, both SUMO-1 and the SUMO-1-conjugating enzyme Ubc9 (10, 46, 47) were found among Mx-interacting proteins in a yeast two-hybrid screen.2 Double immunofluorescence staining of IFN-

2 B. Schumacher and M. Trost, unpublished observations.
treated and PKM-transfected primary mouse embryo cells showed a similar nuclear distribution of PKM and murine Mx1 (Fig. 4B). It remains to be seen whether Mx GTPases and HIPKs are involved in the maintenance of NB structure and function during physiological or pathological cellular processes.

Acknowledgments—We thank Thomas Sternsdorf for Sp100-specific antibodies and helpful discussions and Peter Staeheli, Michael Frese, and Matthias Muller for critically reading the manuscript.

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