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A Novel 205-Kilodalton Testis-Specific Serine/Threonine Protein Kinase Associated with Microtubules of the Spermatid Manchette

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To identify proteins which interact with and potentially modulate the function of microtubules during spermatogenesis, we prepared a total testis MAP (microtubule-associated protein) antiserum and used it to isolate cDNA clones from a mouse testis cDNA expression library. Antibodies affinity purified by using one expression clone recognized a 205-kDa protein, termed MAST205, which colocalizes with the spermatid manchette. Sequencing of full-length cDNA clones encoding MAST205 revealed it to be a novel serine/threonine kinase with a catalytic domain related to those of the A and C families. The testis-specific MAST205 RNA increases in abundance during prepuberal testis development, peaking at the spermatid stage. The microtubule-binding region of MAST205 occupies a central region of the molecule including the kinase domain and sequences C terminal to this domain. Binding of MAST205 to microtubules requires interaction with other MAPs, since it does not bind to MAP-free tubulin. A 75-kDa protein associated with immunoprecipitates of MAST205 from extracts of both whole testis and testis microtubules becomes phosphorylated in in vitro kinase assays. This 75-kDa substrate of the MAST205 kinase may form part of the MAST205 protein complex which binds microtubules. The MAST205 protein complex may function to link the signal transduction pathway with the organization of manchette microtubules.

The process of cellular differentiation is intricately linked to morphological changes in the microtubular cytoskeleton. Microtubules, which form by self-assembly of α- and β-tubulin heterodimers, require specific interactions with various proteins to establish diverse conformations and to perform their different functions. These interacting proteins are referred to as microtubule-associated proteins (MAPs). Two mechanisms can be envisaged for the involvement of MAPs in microtubule morphogenesis. First, the programmed expression of MAPs may be required for the terminal differentiation of a cell type; examples include MAP2 and tau in the determination of neuronal morphology (7). Second, the signal transduction pathway may influence microtubule organization via phosphorylation of tubulin and/or MAPs. Some kinases have been shown to colocalize with microtubules in vivo, providing a means for rapid phosphorylation of specific microtubule proteins. The c-*mos* product, which binds and phosphorylates tubulin (48), is an active component of the cytostatic factor, a factor isolated from mature *Xenopus oocytes* and thought to be responsible for the arrest of fertilized eggs at meiotic metaphase II (31). Among MAPs, phosphorylation of MAP2 has been extensively studied. Phosphorylation of MAP2 reduces its ability to stimulate tubulin assembly in vitro (20, 35) and decreases its affinity for microtubules both in vitro (4) and in vivo (3). Type II cyclic AMP (cAMP)-dependent protein kinase phosphorylates and copurifies with brain MAP2 because of the high-affinity binding of RII to the amino terminus of the MAP2 molecule (38, 44, 45).

Mammalian spermatogenesis is a continuum of cellular differentiation that has three principal phases: the mitotic proliferation and renewal of spermatogonia, the meiotic divisions of spermatocytes, and spermiogenesis, a complex metamorphosis of the haploid germ cell culminating in the release of late spermatids into the lumen of the seminiferous tubule. During spermatogenesis the microtubular cytoskeleton exists in diverse conformations, including two microtubular arrays unique to spermiogenesis, namely the spermatid manchette and flagellum. The manchette temporally forms around the caudal nucleus of round spermatids and has been implicated in the subsequent process of sperm head shaping (9, 32, 39).

We sought to identify proteins which interact with, and potentially modulate the function of, microtubules during spermatogenesis. We describe here the identification and characterization of one such protein, a novel 205-kDa microtubule-associated serine/threonine protein kinase, termed MAST205. MAST205 colocalizes with the microtubular manchette of developing spermatids and may therefore function as a link between the signal transduction pathway, microtubule organization, and sperm head shaping.

MATERIALS AND METHODS

Preparation of microtubule protein. The vasa deferentia, epididymes, and tunica albuginea were removed from two adult bovine testes. The remaining tissue was minced in a meat grinder and then homogenized in a Waring blender for 1 min with 0.25 volume of buffer A (0.2 M MES [morpholineethanesulfonic acid; pH 6.8], 2 mM EGTA, 2 mM $MgCl_2$) containing dithiothreitol (1 mM), GTP (1 mM), leupeptin (10 μg/ml), chymostatin (10 μg/ml), pepstatin (5 μg/ml), phenylmethylsulfonyl fluoride (PMSF) (1 mM), and aprotinin (10 μg/ml). The homogenate was centrifuged at 60,000 × *g* for 40 min, and microtubules in the supernatant were purified by two cycles of temperature-dependent assembly in the presence of 4 M glycerol as described previously (41). Twice-

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cycled calf brain microtubule protein was prepared in a similar manner.

Coassembly of calf brain microtubules and in vitro-translated polypeptides was performed as described previously (27). Sedimentation of microtubules through sucrose cushions was carried out according to the method of Melki et al. (33). For in vitro kinase assays, microtubules were prepared from 20 g of tissue as described above but with the inclusion of the phosphatase inhibitors Na_3VO_4 (200 μM), NaF (20 mM), and sodium PP_i (10 mM).

Tubulin and MAPs were fractionated by chromatography of the microtubule protein on phosphocellulose (47). The MAP fraction was desalted on a column of Sephadex G50.

Generation and affinity purification of antisera. All sera generated in this study were raised in guinea pigs. Sera 339 and 341 were raised against total bovine testis MAPs purified as described above. Sera 659 and 697 were raised against fusion proteins expressed in *Escherichia coli* BL21 DE3 transformed with the pET11 (43)-derived expression vectors, pET412-3 and pET1213-T (see plasmid constructs below), respectively. Preparation of antigens and immunization of animals were carried out as described previously (26).

Monospecific antibodies were purified from immune guinea pig sera as previously described (13) by absorption onto and elution from nitrocellulose strips cut from Western blots (immunoblots) of sodium dodecyl sulfate (SDS) gels containing extracts of *E. coli* expressing MAST205 fusion proteins produced either in BL21 DE3 harboring pET11 recombinants or in Y1089 lysogens infected with bacteriophage λgt11 recombinants (19).

Western blotting. Protein fractions were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (25) and transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline containing 5% defatted milk powder and then incubated with diluted antiserum (serum 697 [1:250] or affinity-purified serum [1 μg/ml]) for 1 h at room temperature. The filters were washed, incubated with ^{125}I-protein A (high specific activity; NEN), washed again, and subjected to autoradiography.

Isolation and analysis of RNA. Total RNA was isolated by the method of Chomczynski and Sacchi (8) from adult (2- to 3-month-old) Swiss Webster mouse brain, heart, intestine, kidney, liver, ovary, spleen, pancreas, stomach, testis, and thymus. Testis RNA was also isolated from 16-day-old embryos, newborn animals, and 7-, 14-, and 21-day-old animals.

For RNA blot transfer, total RNA samples were resolved by electrophoresis in an 0.8% agarose–2.2 M formaldehyde gel, and the gel content was transferred to nitrocellulose in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Prehybridization was performed at 42°C for 3 h in 10× Denhardt's solution (1× Denhardt's solution is 0.02% [each] Ficoll, polyvinylpyrrolidone, and bovine serum albumin)–5× SSC–20 mM sodium phosphate (pH 6.5)–1 mM EDTA–0.1% SDS. cDNA probes were radiolabelled with [α-^{32}P]dATP by nick translation (37). Hybridization was performed at 42°C for 16 to 20 h in 50% (vol/vol) formamide–1× Denhardt's solution–5× SSC–20 mM sodium phosphate (pH 6.5)–1 mM EDTA–0.1% SDS containing ~5 × 10^6 cpm of probe per ml. Filters were washed to a final stringency of 0.5× SSC at 68°C.

Poly(A)+ RNA was purified from adult mouse testis total RNA by chromatography on oligo(dT) cellulose (1).

Isolation and analysis of cDNA clones. Adult mouse testis poly(A)+ RNA was used to prepare cDNA by the method of Gubler and Hoffman (14), using Superscript (Bethesda Research Laboratories) and oligo(dT) or random primers for first-strand synthesis. The cDNA fractions were cloned into either phage λgt10, λgt11, or λGEM2 (Promega). Each library contained approximately 2 × 10^6 independent recombinants.

For antibody screening, approximately 2 × 10^6 random-primed clones in bacteriophage λgt11 were screened as described previously (42), except TBST (50 mM Tris [pH 7.4], 150 mM NaCl, 0.05% Tween 20) containing 5% defatted milk powder was used throughout for blocking the filters. Antisera samples 339 and 341 were used in combination at dilutions of 1:500 and 1:100, respectively. In order to block nonspecific antibody binding, the diluted antibody solutions were preincubated with nitrocellulose filters coated with nonrecombinant bacteriophage λgt11 protein.

For nucleic acid screening, between 5 × 10^5 and 5 × 10^6 oligo(dT)-primed clones in bacteriophage λgt10 or λGEM2 were screened by using 100- to 200-bp fragments derived from the 5′ end (or 3′ end) of cDNA fragments isolated in the previous round of library screening. These fragments were labelled with ^{32}P by nick translation (37). Prehybridization and hybridization conditions were as described above. Filters were washed to a final stringency of 1× SSC at 65°C.

DNA was purified from lytic cultures (10 ml) containing the lambda clones of interest (19).

Primer extension and RACE. Rapid amplification of the 5′ and 3′ ends of the cDNA (5′ and 3′ RACE) encoding MAST205 was performed by minor modification of the published procedure (12), with the following oligonucleotide primers: oligo(dT)$_{17}$ adapter, 5′-GTGGAGGAATTCAAG ATGTGT$_{17}$-3′; adapter, 5′-GTGGAGGAATTCAAGATGT GT-3′; 3′ amplification, 5′-CTGACATCAGATGAGCTCTT-3′; 5′ reverse transcription, 5′-GGACTAAGTCCAGTAAC CATA-3′; and 5′ amplification, 5′-TCCTCCAGTAGCTGC CTC-3′. Superscript (Bethesda Research Laboratories) was used in the reverse transcription reaction mixtures with the buffer supplied by the manufacturer. The reverse transcription reaction mixtures were incubated for 2 h at 37°C, 5 U of enzyme was added, and incubation was continued for 15 min at 50°C. Prior to 5′ RACE, the reverse transcription reaction mixture was extensively dialyzed against TE (Tris-EDTA) buffer in a Centricon 100 (Amicon) centrifugal concentrator. A primer extension reaction, with a mixture of 0.5 fmol of ^{32}P end-labelled 5′ reverse transcription primer and 10 μg of poly(A)+ RNA, was performed as described previously (5) in parallel with 5′ RACE.

Vent DNA polymerase (New England Biolabs) was used throughout for polymerase chain reaction (PCR) amplification. The PCR buffer supplied by the manufacturer was used, supplemented with magnesium sulfate to a final concentration of 5 mM. Hot start PCR was employed. The first PCR cycle (second-strand cDNA synthesis) was conducted at 94°C for 5 min, 52°C for 2 min, and 72°C for 40 min and then followed by 40 cycles at 94°C for 1 min, 52°C for 2 min, and 72°C for 3 min.

The RACE products were treated with DNA polymerase I and polynucleotide kinase and then subjected to digestion with *Eco*RI. The resulting DNA fragments were cloned into pUC19 that had been cleaved with *Eco*RI and *Sma*I. The RACE procedure was repeated on three separate occasions to control for amplification errors, and each product was sequenced.

DNA sequencing. Inserts from lambda clones of interest were excised with *Eco*RI or *Xba*I and subcloned into pGEM7zf+ (Promega). Nested, unidirectional deletions of both strands of the plasmid DNA were generated by using

exonuclease III (18). Plasmid DNA was denatured prior to sequence determination (6) by the chain termination method (40) as described in the Sequenase version 2.0 manual, except the preliminary labelling reaction was omitted. Both DNA strands were sequenced independently.

Sequence data analysis. The Genetics Computer Group software package (11) was used for sequence analyses. Molecular sizes and amino acid compositions were determined by using PEPTIDESORT. FASTA (36) and BLAST (National Center for Biotechnology Information) were used to search the GenBank, SwissProt, and PKCDD (Protein Kinase Catalytic Domain Database) (16) data bases for sequences homologous to MAST205. The program PILEUP was used for multiple pairwise sequence alignments of the kinase domain of MAST205 with the sequences contained in the PKCDD. The programs GAP and BESTFIT were used to align MAST205 with known MAPs.

Plasmid constructs. A full-length cDNA encoding MAST 205 was assembled into the *Eco*RI site of pUC19 from overlapping DNA fragments. This construct, pUCMAST205, was used as the source for all other constructs.

(i) **pET constructs.** pET412-3 was constructed by cloning the 2.4-kbp *Bam*HI-*Bgl*II fragment of MAST205 cDNA into the *Bam*HI site of pET11b (43). pET1213-T was constructed by excising the full-length insert from pUCMAST205 with an *Eco*RI partial digest, filling in the ends with Klenow fragment, ligating with *Bam*HI linkers, cutting with *Bam*HI and *Bgl*II, and cloning the resulting 2.4-kbp fragment into the *Bam*HI site of pET11a.

(ii) **pcDNAI-neo constructs.** The full-length insert from pUCMAST205 was excised with an *Eco*RI partial digest, the ends were flushed with Klenow fragment and ligated with *Xba*I linkers, and the resulting fragment was digested completely with *Xba*I and partially with *Sma*I. The resulting 5.4-kbp fragment was cloned into pcDNAI-neo (Invitrogen) prepared by restriction with *Hin*dIII, flushing the ends with Klenow fragment, and cutting with *Xba*I. This construct, pcDNAMAST205, was used for the preparation of further constructs, as detailed in the legend to Fig. 5, by either deleting the region between two restriction sites or by subcloning MAST205 encoding DNA fragments.

Immunofluorescence microscopy. Cytospin preparations of dissociated mouse testis tissue on poly-L-lysine-coated slides were fixed for 15 min in 4% paraformaldehyde in phosphate-buffered saline (PBS). The fixed cells were permeabilized in 0.2% Triton X-100 in PBS for 5 min and rinsed in PBS. The samples were labelled with affinity-purified anti-MAST205 serum and fluorescein-labelled rabbit anti-guinea pig secondary antibodies. Samples were mounted in 90% glycerol–10% PBS containing 1 mg *p*-phenylene-diamine (21) per ml and examined by using a Zeiss Axiophot fluorescence microscope equipped with a Zeiss Planapochromatic 63× objective.

In vitro transcription and translation. Full-length MAST 205 and MAST205-derived polypeptides were produced in vitro by introduction of the various pcDNAI-neo constructs into a coupled rabbit reticulocyte lysate T7 transcription-translation system (Promega). Translation products were analyzed by SDS–7% PAGE and fluorography.

Immunoprecipitation and immune complex kinase assays. Cell lysates were prepared by homogenizing testis or brain tissue in lysis buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5], 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 0.1 M NaF, 10 mM PP_i, and 0.2 mM Na_3VO_4) (30) containing leupeptin (10 μg/ml), chymostatin (10 μg/ml), pepstatin (5 μg/ml), PMSF (1 mM), and aprotinin (10 μg/ml) in a Dounce homogenizer. Cell lysates were cleared by centrifugation at 100,000 × *g* for 15 min in a Beckman TL100 centrifuge. Cleared lysates (200 μg of total protein) were incubated for 2 h at 4°C with protein A-Sepharose that had been preincubated with either undiluted (5 μl) or affinity-purified (3 μg) anti-MAST205 antibody. The immunoprecipitates were washed extensively with lysis buffer.

For Western blot analysis, the immunoprecipitates were heated to 90°C for 4 min in SDS sample buffer, separated by SDS–7% PAGE, and transferred to nitrocellulose.

For immune complex kinase assays, the immunoprecipitates were washed twice in kinase buffer (50 mM HEPES [pH 7.5], 20 mM $MgCl_2$, 2 mM dithiothreitol), suspended in 50 μl of this buffer containing 0 or 100 μM ATP and 50 μCi of [γ-^{32}P]ATP, incubated for 15 min at 30°C, heated to 90°C for 4 min in SDS sample buffer, and then analyzed by SDS–7 or 8.5% PAGE and autoradiography.

Phosphoamino acid analysis. Phosphoamino acid analysis of labelled proteins eluted from SDS-polyacrylamide gel slices was done by minor modification (29) of the method of Cooper et al. (10).

Nucleotide accession number. The GenBank accession number for the cDNA sequence of MAST205 is U0231.

RESULTS

Isolation and characterization of cDNA clones encoding MAST205. Complementary DNA clones encoding MAST205 were originally identified by screening a random-primed mouse testis cDNA expression library in bacteriophage λgt11 with a polyclonal guinea pig antiserum raised against a total bovine testis MAP preparation. This serum, which recognized multiple species on Western blots of testis microtubule protein (data not shown), detected 28 expression clones. The clones were plaque purified, and the inserts were excised with *Eco*RI and ^{32}P-labelled by nick translation. Each radiolabelled insert was hybridized to DNA from the other clones in a cross-hybridization experiment to reveal the presence of any related sequences. Y1089 lysogens were prepared from the seven classes of clones identified by this criterion. The lysogens were induced with IPTG (isopropyl-β-D-thiogalactopyranoside), and the resulting fusion proteins were resolved by SDS-PAGE and transferred to nitrocellulose filters for use as matrices to selectively purify monospecific sera from the original polyclonal antisera. These sera were used in immunofluorescence localization experiments with cytospin preparations of dissociated mouse testis tissue. Antibodies purified by using the protein expressed by one expression clone, TE6, recognized a 205-kDa protein on Western blots of purified testis MAPs (Fig. 1A). The same antibody detected the manchette of developing spermatids, as revealed by immunofluorescence microscopy (Fig. 1B). We subsequently showed reproducible staining of the manchette (and detection of a 205-kDa protein on Western blots) using the original total MAP serum that had been affinity purified by using other fragments of MAST205 expressed as fusion proteins in *E. coli* and also using sera that had been generated with bacterial fusion proteins encoding, respectively, the C terminus (antiserum 659) and a central region (antiserum 697) of MAST205. MAST205 was not detected in cell types at earlier stages of spermatogenesis. These data suggested a requirement for MAST205 in spermiogenesis with a specific involvement in the microtubular manchette.

Clone TE6 contained a 634-bp insert with a continuous

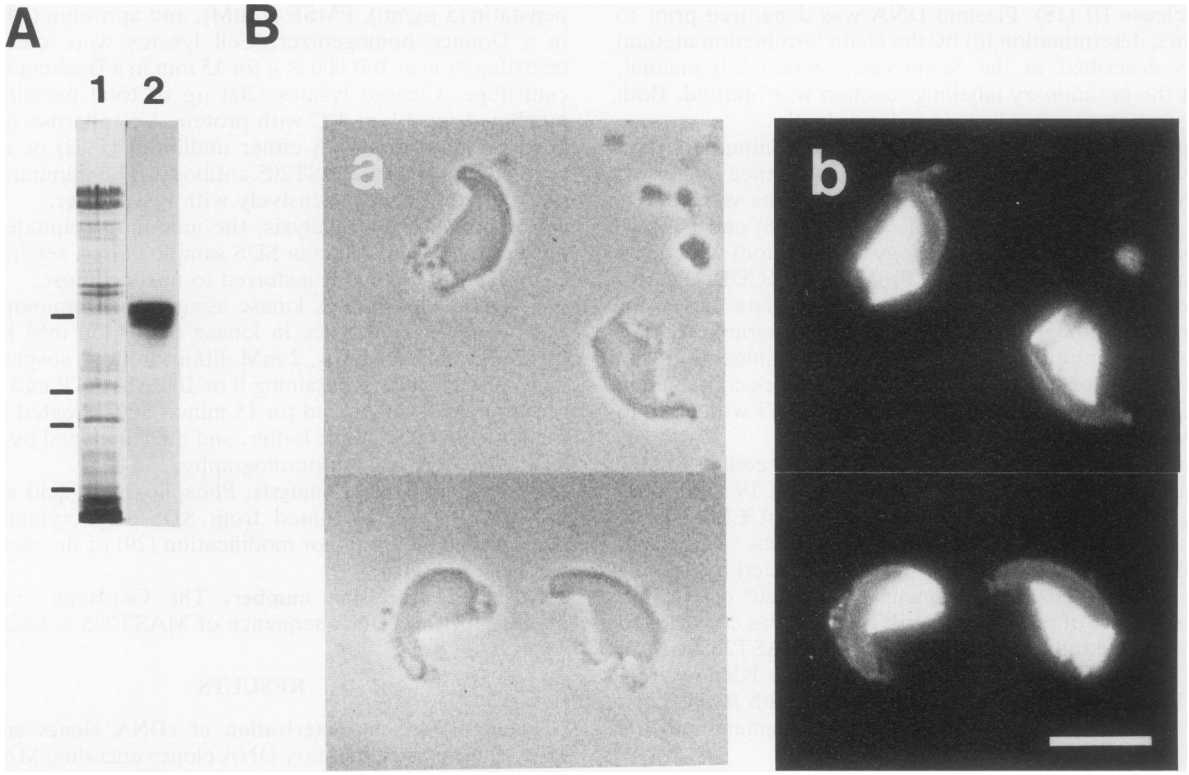

FIG. 1. Western blot and immunofluorescence analysis of antibodies affinity purified by using expression clone λTE6 fusion protein. (A) Bovine testis MAPs (20 μg) were resolved by SDS–5% PAGE and either stained with Coomassie blue (lane 1) or transferred to nitrocellulose and incubated with the TE6 affinity-purified antibody and with ^{125}I-protein A (lane 2). Molecular mass markers (200, 116, 97, and 66 kDa [from top to bottom]) are shown at the left. (B) Photomicrographs of paraformaldehyde-fixed cytospin preparations of dissociated mouse testis tissue showing developing spermatids. (a) Phase-contrast optics; (b) immunofluorescence analysis of the same fields shown in panel a incubated with the TE6 affinity-purified antibody and with fluorescein-labelled secondary antibodies. Bar, 10 μm.

open reading frame. DNA walking experiments were performed in order to isolate overlapping cDNA clones representing the entire MAST205 mRNA. The overlapping cDNA clones include 52 bases of a 3′-nontranslated sequence with a putative polyadenylation signal, AATACA, 14 bp upstream of the poly(A) addition site. The 3′-RACE PCR technique failed to detect heterogeneity in the 3′-nontranslated sequence.

To confirm that the set of overlapping cDNA clones extended to the 5′ end of the corresponding mRNA, a primer experiment using MAST205 mRNA was performed. The single primer extension product (126 nucleotides in length) was amplified by the 5′-RACE technique. The amplified primer extension product contained 27 additional nucleotides 5′ to the set of overlapping cDNA clones. The complete nucleotide sequence of MAST205 is shown in Fig. 2. The first ATG in the sequence, at nucleotide position 117, initiates an open reading frame of 5,202 nucleotides. Two criteria suggest that this initiation codon is used in vivo. First, the sequenced primer extension, 5′-RACE product contained no further upstream ATG codons. Second, sequences surrounding this ATG codon (specifically a purine in the −3 position and a G in the +4 position) provide for efficient translational initiation (24). The next six downstream in-frame methionine codons are in poor context for translational initiation; T7 transcripts produced from constructs in which the first methionine codon was deleted yielded a polypeptide, upon translation in vitro, initiated from ATG 1032 (the seventh in-frame methionine codon) as the overwhelming translation product (data not shown).

The deduced MAST205 polypeptide comprises 1,734 amino acids with a calculated molecular mass of 190,533 Da, slightly less than the apparent molecular mass of 205 kDa deduced by SDS-PAGE. The polypeptide has a calculated pI of 7.69. MAST205 can be subdivided into four domains on the basis of the predicted isoelectric points: a basic (pI = 11.27) N-terminal domain extending from residues 1 to 300, an acidic region (pI = 4.84) from residues 301 to 950, a second basic region (pI = 11.71) extending from residues 951 to 1400, and an acidic (pI = 4.92) carboxy-terminal region from residue 1401. MAST205 contains multiple potential phosphorylation sites for cAMP-dependent protein kinase C and casein kinase II kinases.

Sequence comparison of MAST205 with known proteins. No overall homology of MAST205 to any protein within the current GenBank, EMBL, and SwissProt data bases was found. However, MAST205 contained sequences homologous to the common catalytic domain identified in the protein kinase family (17). Pairwise comparisons of the individual sequences contained within the PKCDD (16) with the homologous region of MAST205 were performed with the program PILEUP. These comparisons indicated that MAST205 is a novel serine/threonine protein kinase related to the A and C families of protein kinases (Fig. 3). In particular, the similarity of the catalytic domain of MAST205 to the catalytic subunits of human cAMP-dependent kinase

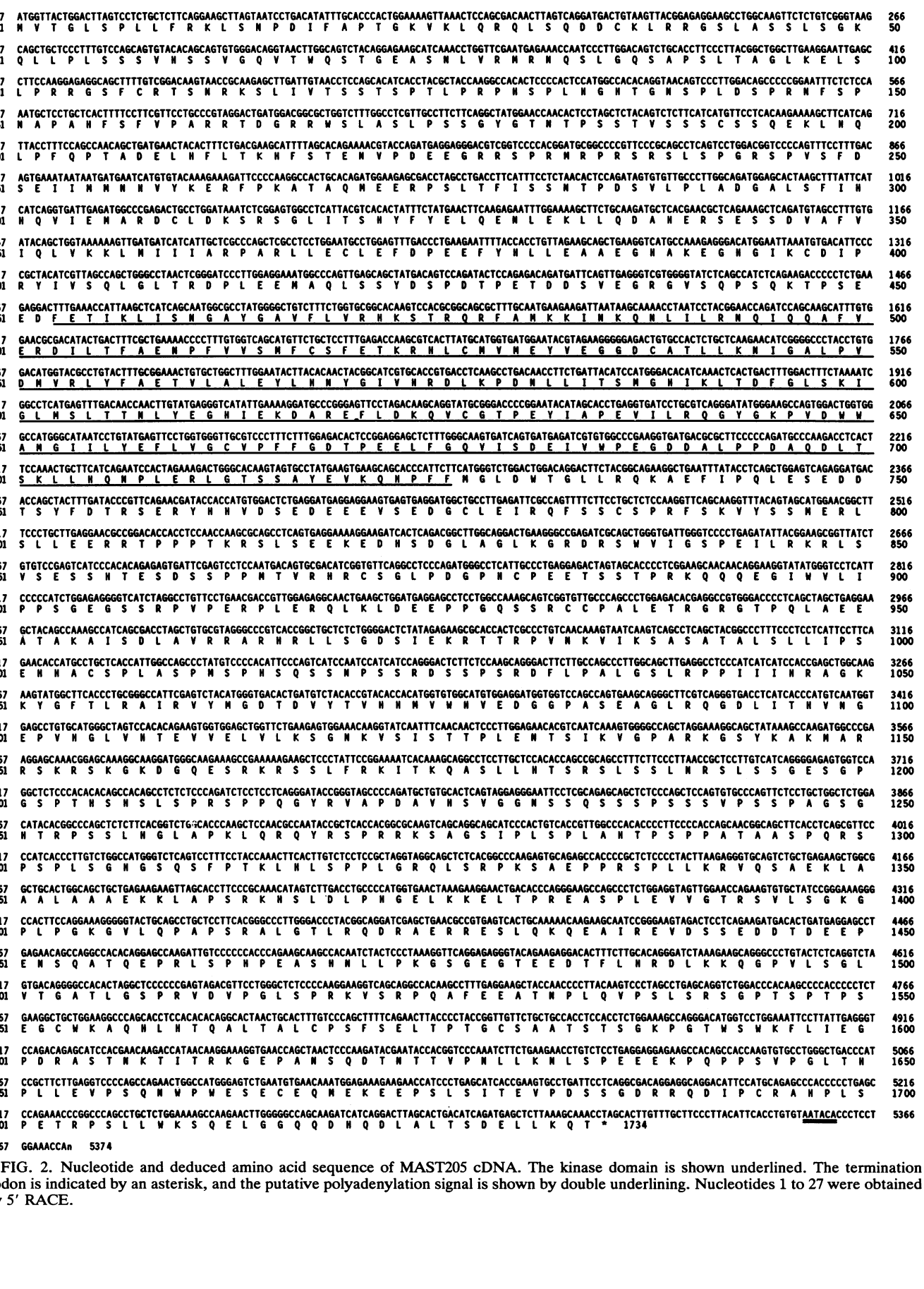

FIG. 2. Nucleotide and deduced amino acid sequence of MAST205 cDNA. The kinase domain is shown underlined. The termination codon is indicated by an asterisk, and the putative polyadenylation signal is shown by double underlining. Nucleotides 1 to 27 were obtained by 5′ RACE.

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Capkα       FERIKTLGTGSFGRVMLVKHKETGNHYAMKILDKQKVVKLKQIEHTLNEKRILQAVN.FPFLVKLEFSFKDNSNLYMVMEYVPGGEMFSHLRRIGRFSEP
MAST205     FETIKLISNGAYGAVFLVRHKSTRQRFAMKKINKQNLILRNQIQQAFVERDILTFAEN.PFVVSMFCSFETKRHLCMVMEYVEGGDCATLLKNIGALPVD
Racα        FEYLKLLGKGTFGKVILVKEKATGRYYAMKILKKEVIVAKDEVAHTLTENRVLQ.NSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLSRERVFSED
Pkcα        FNFLMVLGKGSFGKVMLADRKGTEELYAIKILKKDVVIQDDDVECTMVEKRVLALLDKPPFLTQLHSCFQTVDRLYFVMEYVNGGDLMYHIQQVGKFKEP
consensus   -------g-G----v-----------a-K----------------E-----------------------------------------------------
                  I                  II                III              IV                V

Capkα       HARFYAAQIVLTFEYLHSL.DLIYRDLKPENLLIDQQGYIQVTDFGFAK...RVKGRT..............WTLCGTPEYLAPEIILSKGYNKAVDWW
MAST205     MVRLYFAETVLALEYLHNY.GIVHRDLKPDNLLITSMGHIKLTDFGLSKIGLMSLTTNLYEGHIEKDAREFLDKQVCGTPEYIAPEVILRQGYGKPVDWW
Racα        RARFYGAEIVSALDYLHSEKNVVYRDLKLENLMLDKDGHIKITDFGLCKEGIKDGATM..............KTFCGTPEYLAPEVLEDNDYGRAVDWW
Pkcα        QAVFYAAEISIGLFFLH.KRGIIYRDLKLDNVMLDSEGHIKIADFGMCKEHMMDGVTT..............RTFCGTPDYIAPEIIAYQPYGKSVDWW
consensus   ---------------h------hrD-K--N---------k--Dfg-----------------------------------pE----------D-w
               VIA               VIB               VII                                    VIII       IX

Capkα       ALGVLIYEMAAGYPPFFADQPIQIYEKIVSGKVRFPSH...FSSDLKDLLRNLLQVDLTKRFGNLKNGVNDIKNHKWF
MAST205     AMGIILYEFLVGCVPFFGDTPEELFGQVISDEIVWPEGDDALPPDAQDLTSKLLHQNPLERLGTSS..AYEVKQHPFF
Racα        GLGVVMYEMMCGRLPFYNQDHEKLFELILMEEIRFPRT...LGPEAKSLLSGLLKKDPKQRLGGGSEDAKEIMQHRFF
Pkcα        AYGVLLYEMLAGQPPFDGEDEDELFQSIMEHNVSYPKS...LSKEAVSICKGLMTKHPAKRLGCGPEGERDVREHAFF
consensus   --g----------------------------------------------------R------------h---
                                    X                  XI
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FIG. 3. Amino acid sequence comparison of the kinase domain of MAST205 and those of related kinases. The kinase domains of MAST205, human cAMP-dependent kinase (α-form) (28), rat protein kinase C (α-form) (23), and human racα (22) were aligned by using the Genetics Computer Group program PILEUP. Roman numerals indicate protein kinase subdomains (16). Residues which are invariant (uppercase) or highly conserved (lowercase) in serine/threonine kinase catalytic domains (16) are also shown.

(α-form) (28), rat protein kinase C (α-form) (23), and human racα (22) was 66.0, 66.7, and 66.8%, respectively.

The catalytic domain of MAST205, which extends from residues 453 to 726, contains all the subdomain sequences conserved across the protein kinase family. The sequence motif SNGAYG at positions 460 to 465 (subdomain I) together with the lysine residue at position 482 (subdomain II) conforms well to the protein kinase-specific ATP-binding consensus sequence. Glycine is highly conserved in the first position of the consensus subdomain I sequence GXGXXG. While some kinases which do not contain glycine in this position (e.g., the *Drosophila* gene [*ninaC*] product essential for photoreceptor function [34]) are known, MAST205 represents the first identified protein kinase with a serine residue replacing this glycine. The conserved catalytic domain sequence DFG is also present (amino acids 594 to 596 [subdomain VII]). Two sequences that distinguish serine/threonine-specific and tyrosine-specific protein kinases (15) are also present: DLKPDN (residues 576 to 581 [subdomain VIB]) and TPEYIAPE (residues 627 to 636 [subdomain VIII]).

Outside the catalytic core domain, MAST205 displayed no homology to other known protein kinases or to any other proteins in the current data bases. When MAST205 was compared pairwise with known MAPs by using sensitive homology search matrices, no significant regions of homology were detected, suggesting that MAST205 represented a new type of microtubule-binding protein, the microtubule-binding properties of which are described below.

Expression of MAST205 in mouse tissues. The tissue distribution of MAST205 mRNA was examined by hybridizing total RNA from several mouse tissues with a ^{32}P-labelled 1.6-kbp coding region subfragment of the MAST205 cDNA. A single transcript of 5.5 to 6 kb was detected in testis (Fig. 4A). Hybridization to RNA from brain, heart, intestine, liver, lung, ovary, pancreas, spleen, stomach, and thymus remained undetectable after prolonged exposure. Expression of MAST205 was found to be regulated during testicular development. The transcript, which was virtually undetectable in embryonic day 16 mouse testis, increased in abundance during postnatal testicular development (Fig. 4B). By day 21 the level of expression of MAST205 RNA in testis was equivalent to that seen in the adult (2- to 3-month-old) mouse. In the prepuberal mouse, germ cells reach early and late pachytene stages by days 14 and 18, respectively, with secondary spermatocytes and round spermatids seen in increasing numbers between days 18 and 20 (2). The maximal levels of expression of MAST205 RNA which arise between days 14 and 21 after birth therefore coincide with meiosis/spermiogenesis, consistent with the identification of MAST205 in the spermatid manchette by immunofluorescence microscopy.

Characterization of the microtubule-binding region of MAST205. The region of MAST205 responsible for interact-

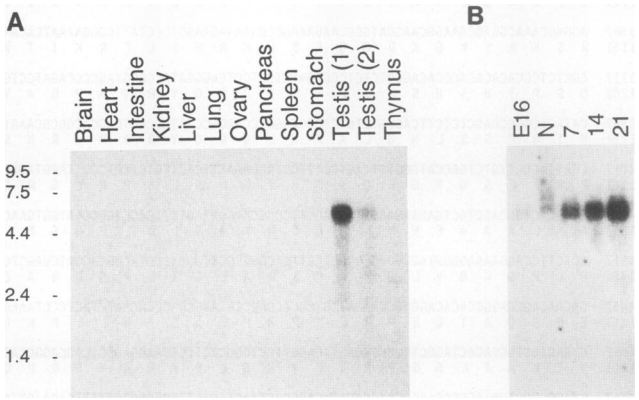

FIG. 4. Expression of MAST205 RNA in mouse tissues. (A) Total RNA was isolated from various tissues from 8-week-old mice and separated by electrophoresis in an 0.8% agarose–2.2 M formaldehyde gel. All gel lanes contained 30 μg of RNA, except the lane labelled testis (2), which contained 3 μg of RNA. The gel content was transferred to nitrocellulose, probed with a 1.6-kbp MAST205 cDNA fragment (see the text), washed, and subjected to autoradiography. Size markers (in kilobases) are indicated on the left. (B) Total testis RNA (30 μg) from embryonic day 16 (E16), newborn (N), and postnatal 7 (7)-, 14 (14)-, and 21 (21)-day-old mice was analyzed as described for panel A.

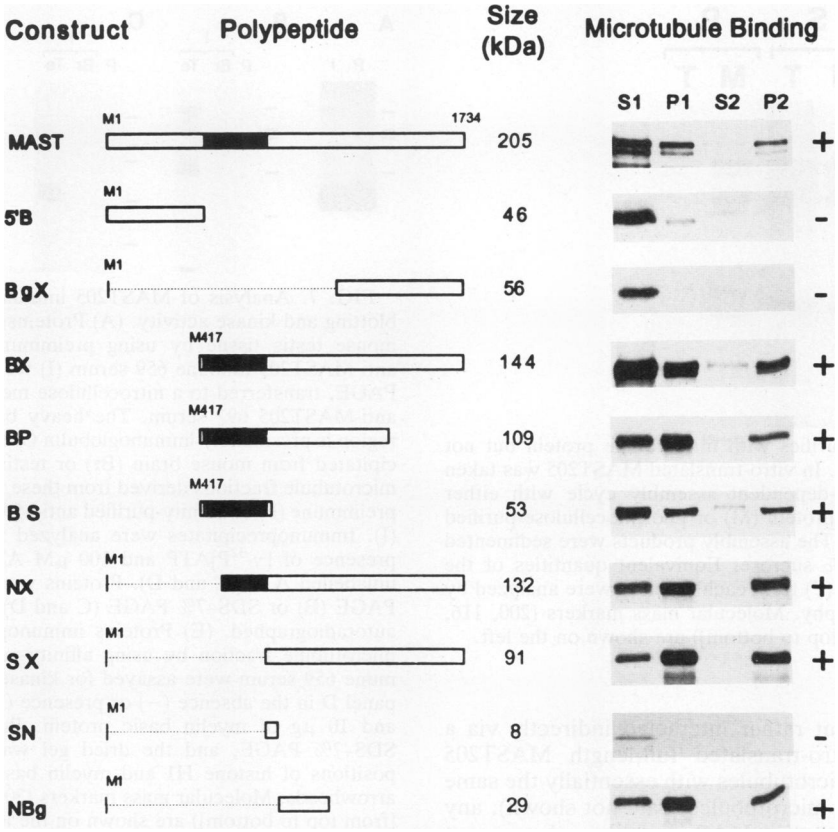

FIG. 5. Localization of the microtubule-binding region of MAST205. pcDNAI-neo expression constructs encoding full-length MAST205, restriction fragments of MAST205, or MAST205 constructs containing in-frame deletions between two restriction sites (indicated by dotted lines) were transcribed and translated in vitro. The extents and sizes of the translated products are shown together with the locations and positions of the initiator methionine codons. The translation products were taken through two cycles of temperature-dependent assembly with carrier calf brain microtubules. Equivalent quantities of the supernatant (S) and pellet (P) from the first (1) and second (2) cycles were subjected to SDS-PAGE and fluorography. The regions of the gels corresponding to the full-length product are shown. Enrichment of the translation product in the microtubule pellet after two cycles of assembly is indicated by +.

ing with microtubules was defined by using a qualitative assay which tested the ability of ^{35}S-labelled, in vitro-translated polypeptide fragments of MAST205 to copurify with microtubules through two cycles of temperature-dependent assembly and disassembly. Figure 5 summarizes the constructs prepared, the corresponding translated polypeptides, and their microtubule-binding properties. With full-length MAST205 (construct MAST), the majority of the translated product which sedimented with microtubules in the first cycle was present in the second-cycle pellet. Enrichment of the translated product in the second-cycle microtubule pellet was the criterion used for microtubule binding. The amino-terminal portion of MAST205 (amino acids 1 to 411; construct 5′ B) did not copurify with microtubules. This protein fragment contains a highly basic region and therefore represents a control for nonspecific electrostatic interactions that might occur with the acidic tubulin subunits. A polypeptide expressed from a construct with an internal deletion encompassing amino acids 2 to 1212 inclusive (construct BgX) also failed to copurify with microtubules, suggesting that the sequences necessary for microtubule binding were contained between amino acids 412 and 1212.

Three constructs in which initiation of protein synthesis occurred from Met-417 were made. Polypeptides produced from these constructs (BX, BP, and BS) (Fig. 5) all copurified with microtubules. Since the polypeptide produced from the BS construct copurified with microtubules, it appeared that the kinase domain of MAST205 was interacting with microtubules. However, deletion constructs that removed amino-terminal sequences including either part (construct NX) or all (construct SX) of the kinase domain also efficiently copurified with microtubules, suggesting that sequences in addition to the kinase domain could also interact with microtubules. The polypeptide produced from construct SN, encoding amino acids 884 to 947 of MAST205, failed to copurify with microtubules, whereas the polypeptide produced from construct NBg did copurify. Thus, in vitro interaction of MAST205 with microtubules can occur via two domains, the kinase domain and a domain between amino acids 948 to 1212. It is possible that in native MAST205 these two domains form a single microtubule-interacting surface or that other sequences contribute to microtubule binding. The sequence of the microtubule-binding region of MAST205 shares no homology with other known MAPs.

Since total microtubule protein (i.e., tubulin and MAPs) was used in the above experiments to define the domains within MAST205 responsible for microtubule binding, we considered the possibility that MAST205 did not interact

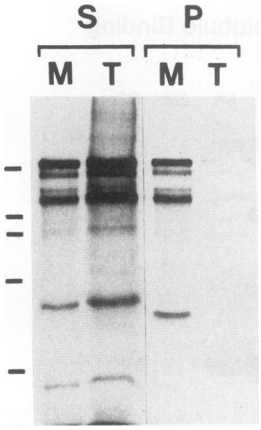

FIG. 6. MAST205 copurifies with microtubule protein but not with tubulin free of MAPs. In vitro-translated MAST205 was taken through one temperature-dependent assembly cycle with either carrier brain microtubule protein (M) or phosphocellulose-purified tubulin free of MAPs (T). The assembly products were sedimented through a cushion of 30% sucrose. Equivalent quantities of the supernatant (S) and pellet (P) from each reaction were analyzed by SDS-PAGE and fluorography. Molecular mass markers (200, 116, 97, 66, and 45 kDa [from top to bottom]) are shown on the left.

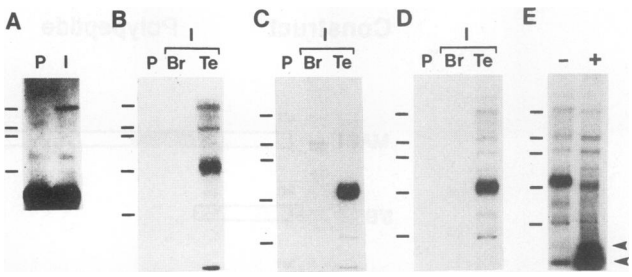

FIG. 7. Analysis of MAST205 immune complexes by Western blotting and kinase activity. (A) Proteins immunoprecipitated from mouse testis tissue by using preimmune (P) or affinity-purified anti-MAST205 immune 659 serum (I) were resolved by SDS–8.5% PAGE, transferred to a nitrocellulose membrane, and probed with anti-MAST205 697 serum. The heavy band in the 50- to 60-kDa region is presumably immunoglobulin G. Proteins were immunoprecipitated from mouse brain (Br) or testis (Te) (B and C) or from microtubule fractions derived from these tissues (D) by using either preimmune (P) or affinity-purified anti-MAST205 immune 659 serum (I). Immunoprecipitates were analyzed for kinase activity in the presence of [γ-^{32}P]ATP and 100 μM ATP (B) or without added unlabelled ATP (C and D). Proteins were resolved by SDS–8.5% PAGE (B) or SDS–7% PAGE (C and D), and the dried gels were autoradiographed. (E) Proteins immunoprecipitated from a testis microtubule fraction by using affinity-purified anti-MAST205 immune 659 serum were assayed for kinase activity as described for panel D in the absence (−) or presence (+) of 10 μg of histone H1 and 10 μg of myelin basic protein. Proteins were resolved by SDS–7% PAGE, and the dried gel was autoradiographed. The positions of histone H1 and myelin basic protein are marked by arrowheads. Molecular mass markers (200, 116, 97, 66, and 45 kDa [from top to bottom]) are shown on the left.

directly with tubulin but rather interacted indirectly via a MAP. Because in vitro-translated full-length MAST205 copurified with testis microtubules with essentially the same efficiency as with brain microtubules (data not shown), any putative MAST205-interacting MAP would have be present in both brain and testis. To address this possibility, ^{35}S-labelled MAST205 was taken through one cycle of temperature-dependent assembly with tubulin either with or without endogenous MAPs, and the assembly products were sedimented through a sucrose cushion. The results of this experiment (Fig. 6) show that MAST205 was enriched in the microtubule pellet fraction only in the presence of endogenous MAPs. The above experiment suggests that MAST205 interacts with microtubules as part of a complex with one or more MAPs.

Immunoprecipitation and kinase activity of MAST205. MAST205 immunoprecipitates were prepared by using nonstringent conditions that would allow proteins interacting with MAST205 (for example, potential activators or substrates of the kinase) to remain associated. Affinity-purified immune serum 659 was used for immunoprecipitation since this antibody interacts with a region of MAST205 C terminal to the kinase or microtubule-interacting domains. Proteins immunoprecipitated from mouse testis were resolved by SDS-PAGE and analyzed by Western blotting with antiserum 697 (reactive towards the central region of MAST205, including the kinase domain). The results (Fig. 7A) show immunoprecipitation of a 205-kDa protein from mouse testis with the affinity-purified antibody but not with preimmune serum.

When MAST205 immune complexes from mouse testis were incubated with 100 μM [γ-^{32}P]ATP and analyzed by SDS-PAGE and autoradiography, labelled protein species with sizes of 205, 110, and 75 kDa were observed (Fig. 7B). Antiserum 697, which effectively immunoprecipitates in vitro-translated, full-length MAST205, was neutralizing in these in vitro kinase reactions, such that phosphorylation of the 205-, 110-, and 75-kDa species was ablated. No radioactive species were seen in immune complexes obtained from mouse testis by using preimmune serum or in immune complexes obtained from mouse brain (in which MAST205 is not expressed) by using immune serum. The 205-kDa protein presumably represents phosphorylation (possibly autophosphorylation) of MAST205. The 110- and 75-kDa proteins may represent either specific substrates associated with MAST205 or breakdown products of MAST205 that retain autophosphorylation activity or nonspecific proteins bound to immunoglobulin G, which act as substrates of MAST205. While we did not attempt to distinguish between these possibilities, the 75-kDa protein and, to a lesser extent, the 110-kDa protein at least served as convenient markers of kinase activity. Incorporation of phosphate into the 75-kDa protein was increased relative to incorporation into the 205- and 110-kDa proteins when cold ATP was omitted from the kinase assays (Fig. 7C), suggesting differences in the phosphorylation of the three proteins. Since MAST205 copurifies with microtubules, we also analyzed kinase activity in MAST205 immune complexes obtained from testis microtubule protein (Fig. 7D). A 75-kDa protein was the major phosphorylated product, with additional weakly phosphorylated proteins with sizes of 205-kDa or less (possible degradation products of MAST205 resultant from cycles of microtubule polymerization). No major phosphorylated species were seen by using preimmune serum or in MAST205 immune complexes obtained from brain microtubules. One possibility is that the 75-kDa protein forms part of a microtubule-binding complex with MAST205.

The above results indicated that the kinase activity of MAST205 immunoprecipitates phosphorylates a 75-kDa protein associated with the immune complex. This kinase activity was apparently specific to MAST205, since no

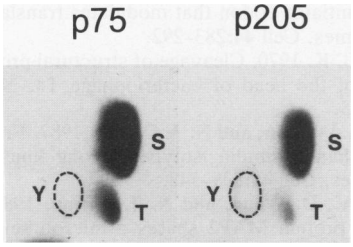

FIG. 8. Serine/threonine kinase activity associated with MAST 205 immunoprecipitates. The ^{32}P-labelled 205-kDa (p205) and 75-kDa (p75) phosphoproteins seen after SDS-PAGE of MAST205 immune complex kinase assays (Fig. 7B) were eluted from gel slices and hydrolyzed. The hydrolysates were mixed with nonradioactive phosphoamino acid markers and then analyzed by two-dimensional thin-layer chromatography and autoradiography. The migrations of the markers are indicated. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

incorporation of phosphate into protein was seen in immunoprecipitates from testis by using preimmune serum or in immunoprecipitates from brain by using immune serum (Fig. 7B, C, and D). Incubation of myelin basic protein and histone H1 with the MAST205 immune complexes obtained from testis microtubules resulted in incorporation of phosphate into these substrates, concomitant with decreased phosphorylation and increased electrophoretic mobility of the 75-kDa protein (Fig. 7E), suggestive of substrate competition. We conclude from these data that the kinase activity associated with MAST205 is capable of phosphorylating a 75-kDa protein in vitro which may be a protein substrate (possibly a MAP) associated with MAST205 in vivo.

The predicted amino acid sequence of MAST205 indicated it to be a serine/threonine-specific kinase. To address the specificity of the kinase activity associated with MAST205 immunoprecipitates, the in vitro-phosphorylated 205- and 75-kDa proteins (Fig. 7B) were subjected to phosphoamino acid analysis. The results shown in Fig. 8 indicate serine/threonine kinase activity (but no tyrosine kinase activity). The serine kinase activity associated with MAST205 immunoprecipitates is significantly greater than the threonine kinase activity.

DISCUSSION

We report here the identification and characterization of a novel testis-specific serine/threonine protein kinase, MAST 205, which copurifies with microtubules in vitro and colocalizes with the spermatid manchette by immunofluorescence microscopy. The 274-amino-acid catalytic domain is related to those of the A and C families of protein kinases (Fig. 3). Included in the catalytic core of MAST205 is the subdomain I sequence SNGAYG, with serine replacing the highly conserved glycine in the first position. The microtubule-binding region of MAST205 resides in the central portion of the molecule, encompassing the kinase domain and sequences C-terminal to this domain up to amino acid 1212. The microtubule-binding domain of MAST205 differs from those of known MAPs, but this is not surprising since the binding of MAST205 to microtubules seems to require other proteins (MAPs). The functions of those sequences in MAST205 N terminal to the kinase domain and C terminal to the microtubule-binding region are not known. The amino terminus of MAST205 may be a kinase regulatory region analogous to the protein kinase C (PKC) and cGMP-dependent kinase families. MAST205 contains multiple putative phosphoryl acceptor sites which conform to recognition sequences for cAMP-dependent and PKC kinases. Many of these potential phosphoryl acceptor sites are clustered within the microtubule-binding region of MAST205 C terminal to the kinase domain and therefore may be involved in the regulation of microtubule binding and/or kinase activity. These sites are apparently phosphorylated in vivo, since serum 697 (raised against an *E. coli* fusion protein containing this region of MAST205) reacted more strongly by immunofluorescence of testis cytospin preparations when the fixed tissue was pretreated with alkaline phosphatase (46).

The significance of the manchette in mammalian spermiogenesis has been inferred from ultrastructural observations of conditions which perturbed the manchette (by using chemical agents or in mutant mice) and consequently affected nuclear shaping (10, 32, 39). In an attempt to determine the contribution of MAST205 to manchette structure and function, we transiently transfected Cos cells with a full-length construct encoding MAST205 cloned into the mammalian expression vector pcDNAI-neo. Transcription from the T7 promoter of this vector produced RNA competent for translation of full-length MAST205 in vitro (Fig. 5). However, we failed to detect MAST205 expression in Cos cells transfected with this construct (46). While the functional significance of MAST205 expression in spermiogenesis remains to be determined, one possibility is that through the signal transduction pathway, MAST205 may regulate the formation, movement, or disassembly of the manchette during spermiogenesis.

MAST205 may function in the manchette by regulating the behavior of MAPs bound to microtubules, since MAST205 copurifies efficiently with microtubules containing MAPs but not with MAP-free microtubules. The copurification of the testis-specific MAST205 protein with both brain and testis microtubules strongly implies that one or more MAST205-interacting proteins are present in both tissues. While the exact composition of the MAST205 complex is unknown, fractions containing the majority of MAST205 and its associated kinase activity elute with a molecular mass of 2×10^6 to 3×10^6 Da when extracts of mouse testis are chromatographed on Superose 6 (46); the minimum size that retains kinase activity is $\sim 10^6$ Da. A candidate MAST205-interacting protein is the 75-kDa phosphoprotein consistently seen associated with MAST205 immunoprecipitates from extracts of whole testis or testis microtubule fractions. This 75-kDa protein becomes heavily phosphorylated by the kinase activity associated with the MAST205 immune complex (Fig. 7) and may therefore be an enzyme-bound substrate of the MAST205 kinase.

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