Male-specific IDGF, a Novel Gene Encoding a Membrane-bound Extracellular Signaling Molecule Expressed Exclusively in Testis of Drosophila melanogaster*

We identified a novel gene of Drosophila melanogaster, Male-specific IDGF (MSI), encoding a transmembrane signaling molecule with exclusive expression in the testis. This molecule (MSI) contains a single transmembrane domain and has 35% amino acid identity with the testis. This molecule (MSI) contains a single transmembrane signaling molecule with exclusive expression in rest mutants, developmental stages. Analysis using four meiotic arrests showed that MSI mRNA was restricted to mature primary spermatocytes, whereas MSI was detected in the cells at the later developmental stages. Analysis using four meiotic arrest mutants, aly, can, mia, and sa suggested that MSI is involved in spermiogenesis, the final differentiation step of spermatogenesis. These results suggest that MSI is an extracellular signaling molecule participating in spermatogenesis and is a new member of the IDGF family.

Spermatogenesis is a complex multistep differentiation program leading to the development of highly specialized haploid male gametes from primordial diploid cells. This differentiation program starts with the formation of spermatogonia from germ line stem cells followed by mitotic divisions to produce primary spermatocytes. Primary spermatocytes then undergo meiotic divisions to give rise to haploid spermatid cells followed by the final differentiation step, spermiogenesis, to produce motile spermatozoa.

In Drosophila, the process of spermatogenesis has been extensively studied at the cellular level (1–3), and genetic analysis has revealed several loci that are required for the spermatogenic processes (3–5). Furthermore, recent molecular biological studies have identified several genes that function in spermatogenesis, including β2-tubulin, twine, boule, degenerative spermatocyte-1 (des-1), and fuzzy onions (fzo). The gene product of β2-tubulin is one of the major components of the germ cell-specific cytoskeleton and is essential for normal germ cell development (6). Other genes, twine, boule, and des-1, are known to be required for the meiotic division. The twine gene encodes a Drosophila homologue of cdc25, a cell-cycle phosphatase, which is essential for the onset of the first meiotic division (7–9). The boule gene encodes a predicted RNA-binding protein showing strong homology with the product of the human DAZ gene, a candidate for the Y-chromosome azoospermia factor (10), whereas the des-1 gene product is a novel membrane protein required for the initiation of meiosis (11). The fzo gene encodes a protein with GTPase motifs and regulates mitochondrial fusion at the early postmeiotic stage (12). In contrast to the extensive studies of spermatogenesis at the gene level, the upstream regulatory factors for these genes, such as extracellular signaling molecules, have not been well characterized.

Insect-derived growth factor (IDGF)* is the first soluble invertebrate growth factor to be identified, being purified from the conditioned medium of an embryonic cell line of the flesh fly, Sarcophaga peregrina (13). This factor had no significant sequence similarity with any other growth factors so far characterized, but did show homology with atrial gland granule-specific antigen (AGSA) of Aplysia californica (about 25% sequence identity), a secretory protein with unknown function (14). Here, we describe the identification of a novel Drosophila gene, Male-specific-IDGF (MSI), which is expressed exclusively in testis and encodes a protein termed MSI, which is assigned as a novel member of the IDGF family. Data suggest that MSI is an extracellular signaling molecule required for spermatogenesis.

EXPERIMENTAL PROCEDURES

cDNA Cloning of MSI—PCR was done using Drosophila melanogaster genomic DNA as a template. DNA primers were designed from amino acid sequences of Sarcophaga AGSA that showed relatively strong homology with Aplysia AGSA. The nucleotide sequences of the primers for the initial PCR were 5′-TA(T/C) ATG AGG CC(T)/ C/A/G AA(A/G) GG-3′ and 5′-CCI GC(A/G) TCI GTG(A/G) TA(T/C) TT-3′, which correspond to the IDGF amino acid sequences of YISSMFKG134 and KYPDFVAG345, respectively. The primers for the nested PCR were 5′-ATI AGI AG1 ATG CC(T)/ C/A/G AA(A/G) GG-3′ and 5′-TA(T/C) TI ATG CC(A/G) TT AT (A/G) AA(A/G) TC-3′, which correspond to HLYTDRN508 and HLYPDRN508, respectively. The 620-bp DNA fragment of the nested PCR product was amplified. The sequence analysis showed that it encoded a peptide with 162 amino acid residues split by a 132-bp intron.

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Tests-specific Extracellular Signaling Protein

Because this peptide showed significant sequence homology with part of IDGF, this 620-bp DNA fragment was used as a probe for subsequent cDNA cloning. The probe was labeled with [α-32P]dCTP using a random primer labeling kit (Takara, Kyoto). One hybridization-positive clone was isolated from 1.5 × 107 clones of a Drosophila adult cDNA library (Stratagene) by plaque hybridization, and the complete sequence was determined. Data base searches were done using the BLAST program (Stratagene) by plaque hybridization, and its complete sequence was deposited in the GenBank database. Searches were done using the BLAST program (Stratagene) by plaque hybridization, and its complete sequence was deposited in the GenBank database.

A plasmid that drives the expression of MSI was constructed by amplifying the DNA fragment of MSI cDNA (nucleotides 1 to 1686 in Fig. 1A) by PCR and ligating the PCR product downstream of the Drosophila Actin 5C promoter fragment. Direct subcloning of the plasmid into the Schneider’s line 2 cells was performed as described by Hishihara et al. (18). Briefly, cells were seeded at a density of about 3.5 × 105 cells/well in a 6-well plate and cultured overnight. The medium was then changed to serum-free medium (Life Technologies, Inc.). The constructed plasmid DNA (1 μg) and 4 μl of Cellfectin reagent (Life Technologies, Inc.) were mixed. The mixture was added to each well, and incubation was continued in medium that was then changed to serum-free medium. Schneider’s insect medium and cultured for a further 2 days. The expression of MSI was examined by immunodetection. About one-tenth of the cells was transfected reproducibly under these conditions.

Preparation of Antibody against MSI—A 50-μg aliquot of the synthetic peptide CVDEEFYNLWRNYHSQP was conjugated to keyhole limpet hemocyanin and injected into an albino rabbit with complete Freund’s adjuvant. Three booster injections were given before harvesting the antiserum. In this peptide, V to P corresponded to residues 119 to 144 (Pro) of MSI. The N-terminal Cys was added to couple this peptide to Sepharose in preparation for purification of the antibody by affinity column chromatography.

Cell Fractionation and Immunoblotting—Fractionation of the transfected Schneider’s line 2 cells was performed as follows: cells were homogenized in 10 volumes of phosphate-buffered saline (PBS) containing 0.5% (v/v) heat-inactivated fetal bovine serum (FBS), streptomycin (0.5 mg/ml), and penicillin G (120 units/ml) at 27 °C.

Immunoblotting was performed essentially as described by Burnette (19). Briefly, proteins separated by SDS-polyacrylamide gel electrophoresis were transferred onto a polyvinylidene difluoride membrane (19). Briefly, proteins separated by SDS-polyacrylamide gel electrophoresis were transferred onto a polyvinylidene difluoride membrane. The resulting filter was then treated with 0.5 μg/ml anti-MSI antibody solution followed by peroxidase-linked anti-rabbit donkey IgG diluted 1:5000. The filter was then treated with ECL Western blotting detection reagents (Amersham Pharmacia Biotech) and exposed to x-ray film.

Immunofluorescence Staining—Immunofluorescence staining of the transfected Schneider’s line 2 cells under non-fixative conditions was carried out as described by Hori et al. (20). Briefly, the cells were suspended in 10 ng/ml anti-MSI antibody solution and kept on ice for 1 h. They were then washed three times with PBS containing 10% (v/v) FBS, and suspended in FITC-labeled secondary antibody solution for 40 min. The cells were then washed, transferred to 12-well multitest slides, and examined with a fluorescence microscope. Immunofluorescence staining under non-fixative conditions was performed as described by Hishihara et al. (18). Briefly, the transfected cells on coverslips were fixed in 4% (v/v) paraformaldehyde in PBS for 10 min and blocked in 1% (v/v) skim milk, 0.1% (v/v) Triton X-100 in PBS for 10 min. Then the cells were successively treated with 10 ng/ml anti-MSI antibody and FITC-labeled secondary antibody (Dako Corp.). Then they were washed three times with PBS, mounted in 50% (v/v) glycerol containing 2.5% (v/v) 1,4-diazobicyclo[2.2.2]octane, and their fluorescence was examined under a fluorescence microscope.

For whole mount immunostaining of testes, testes from adult flies were fixed with 4% (v/v) formaldehyde, 1% (v/v) Nonidet P-40, 0.1% (v/v) Triton X-100 in PBS for 1 h, then followed with the protocol as described above. We used yw strain in immunofluorescence study because of its low level of natural fluorescence.

Assay for the Growth Factor Activity—Large scale preparation of transfected Schneider’s line 2 cells for the measurement of the growth factor activity of MSI was achieved by using a 75-cm2 tissue culture flask. Assay of the growth factor activity was carried out as described before (13). The transfected Schneider’s line 2 cells were suspended in serum-containing Schneider’s insect medium at a density of 106 cells/ml, and 100 μl of the cell suspension was poured into each well of a 96-well microculture plate. The plates were incubated at 27 °C.

Because this peptide showed significant sequence homology with part of IDGF, this 620-bp DNA fragment was used as a probe for subsequent cDNA cloning. The probe was labeled with [α-32P]dCTP using a random primer labeling kit (Takara, Kyoto).
is a membrane-bound extracellular signaling molecule and a novel member of the IDGF family rather than an IDGF homologue of *Drosophila*.

**Expression of MSI in Schneider’s Line 2 Cells**—Because MSI was suggested to be a transmembrane protein, we examined its cellular localization by transiently expressing it in Schneider’s line 2 cells, which have no endogenous expression of MSI. For this, MSI cDNA was expressed in these cells under the control of the *Drosophila* actin 5C promoter, and the cells were subjected to immunodetection using a specific antibody raised against the peptide derived from MSI. Results from the immunoblotting experiment are shown in Fig. 2A.
exclusively in the membrane fraction, when the cells were fractionated into the membrane and cytosolic fractions, while co-expressed β-galactosidase, which is a cytosolic protein, was recovered in the cytosolic fraction. The culture medium did not contain an appreciable amount of MSI, indicating that it is not a secretory protein (data not shown). Furthermore, immuno-
fluorescence staining of the transfected cells under non-fixative conditions revealed that this protein is localized on the cell surface (Fig. 2, B and C). When fixed cells were stained with the same antibody, patches were detected along the edges of the cells (Fig. 2, D and E). These results support the conclusion that MSI is a transmembrane protein with its C-terminal side forming an extracellular domain, because the epitope recognized by the antibody used here is located in this domain.

**Growth Factor Activity of MSI**—We next examined whether MSI exhibits growth factor activity, like IDGF. For this, [methyl-³H]thymidine incorporation of MSI-transfected Schneider's line 2 cells was compared with that of the control cells. As shown in Fig. 3, thymidine incorporation of MSI-transfected cells was significantly higher than that of the mock transfected cells at a cell density of 10⁷ cells/ml (p < 0.01), where the cells were confluent. Almost the same results were obtained with medium containing 5% and 12% FBS when cell density was 10⁷ cells/ml. In contrast, no appreciable enhancement of cell growth was detected at lower cell densities. Possibly, contact of membrane-bound MSI to adjacent cells may not be sufficient under these conditions. In addition, no growth factor activity was detected in the conditioned medium of the transfected cells (data not shown). These results suggest that MSI in fact exhibits growth factor activity through cell-to-cell interaction. The enhancement rate of 1.5- to 2-fold may be reasonable, considering the fact that the ratio of MSI-expressing cells is about 10% and that MSI is a transmembrane protein.

**MSI Is a Testis-specific Protein**—To determine when and where MSI functions, we performed gene expression analyses. Northern blot analysis throughout the life cycle of *Drosophila* revealed that the MSI mRNA is expressed in pupae and adult males, in association with the development of adult organs (Fig. 4). The signal detected in pupae is likely to be from males, because its expression in the adult is restricted to the male. These results suggest that the tissue synthesizing MSI is testis. Results of RT-PCR to test whether expression is testis-specific or not are shown in Fig. 5. MSI mRNA was detected in the testes of adult males but not in the carcass after removal of the testes, indicating that *MSI* is expressed exclusively in testis.
To investigate the cells expressing this molecule, in situ hybridization with testis squashes was performed. Fig. 6 shows that the mature primary spermatocytes are the cells that express MSI mRNA (Fig. 6B, open arrowheads). However, the signal was absent in the apical tip of the testis, where the stem cells and spermatogonial cells are localized. Nor was the signal detected in the developing primary spermatocytes that are localized between spermatogonial cells and primary spermatocytes (Fig. 6B, solid arrowheads). No such signals were detected with a sense probe (Fig. 6C).

Next, the distribution of MSI was examined by immunofluorescence staining. Testes were successively treated with antibody and FITC-conjugated second IgG. The specificity of the antibody was confirmed by immunoblotting, where it gave a single band with testis homogenate (Fig. 7A). In contrast to in situ hybridization result, fluorescence was detected in the regions where spermatocytes at much later developmental stages, including spermiogenesis, are present (Fig. 7B). Because the large number of gene products needed for post-meiotic stages are thought to be supplied in the mature primary spermatocytes by premeiotic transcription (2, 3, 25, 26), MSI is likely to be a protein that functions in meiosis or spermiogenesis.

Expression of MSI in Meiotic Arrest Mutants—To examine the relation between MSI and other genes, we investigated the expression level of MSI mRNA in four meiotic arrest mutants, aly6, can3, mia1, and sa1. Lin et al. (27) and White-Cooper et al. (28) showed that the transcription of the genes involved in spermiogenesis, such as fzo (12) and don juan (24) are greatly reduced in all these four mutants, whereas that of the genes required for meiosis, such as twist (7–9) and boule (10) are not affected in the aly6 mutant. As shown in Fig. 8, no appreciable transcript of MSI was detected in any of the four mutants,
DISCUSSION

We identified a new member of the IDGF family from Drosophila. Although MSI is a membrane-bound protein, it is structurally related to secretory IDGF and AGSA, suggesting that these three proteins belong to a common structural family. Of these, IDGF was shown to be a growth factor of Sarcophaga embryonic cells. Possibly, IDGF interacts with a specific receptor on the surface of these cells and stimulates their growth. By contrast, MSI was suggested to be a transmembrane protein consisting of a 47-residue intracellular domain and a long C-terminal extracellular domain with a 17-residue transmembrane domain. By analogy with IDGF, MSI is likely to be a membrane-bound signaling molecule and possibly interacts with other cells with its extracellular domain. In fact, MSI expressed in Schneider’s line 2 cells was shown to localize on the surface and exhibits growth factor activity when cells are kept in high density. These results suggest that Schneider’s line 2 cells contain receptor(s) for MSI.

Gene expression studies revealed that MSI is a testis-specific protein, and its mRNA was detected exclusively in mature primary spermatocytes ready to enter into meiosis. Because most transcription in spermatocytes is shut off upon entry into meiotic division in spermatogenesis of Drosophila, it is known that mRNAs for a large number of genes required for postmeiotic spermatid differentiation are synthesized by premeiotic transcription and deposited in mature primary spermatocytes (2, 3, 25, 26). In accordance with these previous reports, MSI was shown to be expressed in the spermatocytes at the later developmental stages, suggesting that MSI functions in postmeiotic differentiation. Although MSI exhibits growth factor activity to embryonic cells such as Schneider’s line 2 cells, it might convey a signal in the testis, because the cells at those developmental stages are differentiating rather than proliferating.

The evidence that MSI is not expressed in meiotic arrest mutants so far tested suggests that it is likely to be a spermiogenic gene, according to the proposal of White-Cooper et al. (28). This is consistent with the expression pattern of MSI. The facts that MSI is a testis-specific protein and its transcription is under the control of meiotic arrest genes suggest that MSI plays a role in spermiogenesis.

In spermatogenesis of Drosophila, developing germ cells are surrounded by cyst cells, and differentiation of the germ cells progresses in this delimited space (30, 31). Although the cyst cells are required for the differentiation of the germ cells, the germ cells are also known to regulate the fate of the cyst cells. This close association of the germ cells and cyst cells suggests the presence of signaling processes between these two cells. On the other hand, signaling processes could also be present between two germ cells. However, no molecules have been identified that mediate cell-to-cell interaction in spermatogenesis. Judging from its IDGF-like structure and in vitro activity, MSI could be a candidate for such a molecule on the surface of postmeiotic spermatids, which transmits a specific signal to adjacent cells to bring them into spermiogenesis.

The evidence described here raises the possibility that MSI has a function in spermiogenesis as an extracellular signaling molecule. To clarify the biological role of MSI in spermatogenesis, a loss-of-function mutation of the MSI gene would be helpful, and such strains could be generated by the technique of
insertional mutagenesis with single P elements (32, 33). The identification of the receptor for MSI, if any, would be also helpful to understand the function of MSI. We found three genes related to MSI in the data base of Drosophila genome. Those genes are likely to be for the new members of IDGF family, but their functions are unknown.

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