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Physical Interactions of Dmnk with Orb: Implications in the Regulated Localization of Orb by Dmnk during Oogenesis and Embryogenesis

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The *Dmnk* (*Drosophila* maternal nuclear kinase) gene, encoding a nuclear protein serine/threonine kinase, is expressed predominantly in the germline cells during embryogenesis, suggesting its possible role in the establishment of germ cells. We report here that Dmnk interacts physically with *Drosophila* RNA binding protein Orb, which plays crucial roles in the establishment of *Drosophila* oocyte by regulating the distribution and translation of several maternal mRNAs. Considering similar spatiotemporal expression pattern of *Dmnk* and *orb* during oogenesis and early embryogenesis, it is suggested that Dmnk plays a role in establishment of germ cells by interacting with Orb. Although there are two forms of Dmnk proteins, Dmnk-L (long) and Dmnk-S (short) via the developmentally regulated alternative splicing, Orb can associate with both forms of Dmnk proteins when expressed in culture cells. However, immunohistochemical analysis revealed that Dmnk-S, but not Dmnk-L, can affect the subcellular localization of Orb in a kinase activity-dependent manner, suggesting differential functions of Dmnk-S and Dmnk-L in the regulation of Orb.

Key Words: **nuclear protein kinase; RNA binding protein; maternal gene; *Drosophila melanogaster*.**

In many organisms, the proper spatial distribution of cytoplasmic determinants in eggs and oocytes is essential for embryonic patterning (1, 2). These determinants have been well characterized in *Drosophila* (3–5). The posterior determination of the *Drosophila* embryo is controlled primarily by a series of maternal effect genes, called as posterior group genes. Most of these genes are also required for the proper localization and assembly of the germ-line determinants at the posterior pole of the oocyte. The localization of the mRNAs transcribed from these genes to precise sites are required for the development of the abdomen and formation of the precursors of germ cells (pole cells) in *Drosophila* (2, 6, 7).

One of the *Drosophila* maternal genes, *Dmnk* (*Drosophila* maternal nuclear kinase), encoding a nuclear protein serine/threonine kinase, exhibits restricted expression pattern during oogenesis and embryogenesis. *Dmnk* transcript is detectable from very early stages during oogenesis, and subsequently localized in the anterior of oocytes during oogenesis, in a manner similar to several maternal transcripts that are required for oogenesis and early embryogenesis (8). There are two forms of Dmnk proteins, Dmnk-L (long) and Dmnk-S (short) proteins, as a result of the alternative splicing. It has also been shown that expression of these two transcripts is developmentally regulated, but the functional difference between Dmnk-L and Dmnk-S is still unknown (8). Dmnk is the *Drosophila* homolog of Rad53 (*Saccharomyces cerevisiae*), Cds1 (*Schizosaccharomyces pombe*), Ce-cds-1 (*Caenorhabditis elegans*), and Chk2 (mammals) (9–14). These protein kinases possess a phosphospecific protein-protein interaction motif, the forkhead-associated (FHA) domain (15). It has been shown that these kinases are required for various cellular processes, including cell cycle checkpoint regulation, DNA repair, and meiotic recombination (10–12, 16–19). Although the functions of Dmnk during oogenesis and embryogenesis remain largely unknown, the spatiotemporal distribution of *Dmnk* in ovaries and early embryos suggests its possible function(s) in characteristic features of germ cells such as meiosis and/or germline establishment. It can be assumed that the isolation of a protein(s) interacting with Dmnk protein will open a way to clarify the function of Dmnk during development.

The spatiotemporal expression patterns of *Dmnk* mRNAs are quite similar to those of *orb* transcripts (20–22). The *orb* gene encodes a protein containing two

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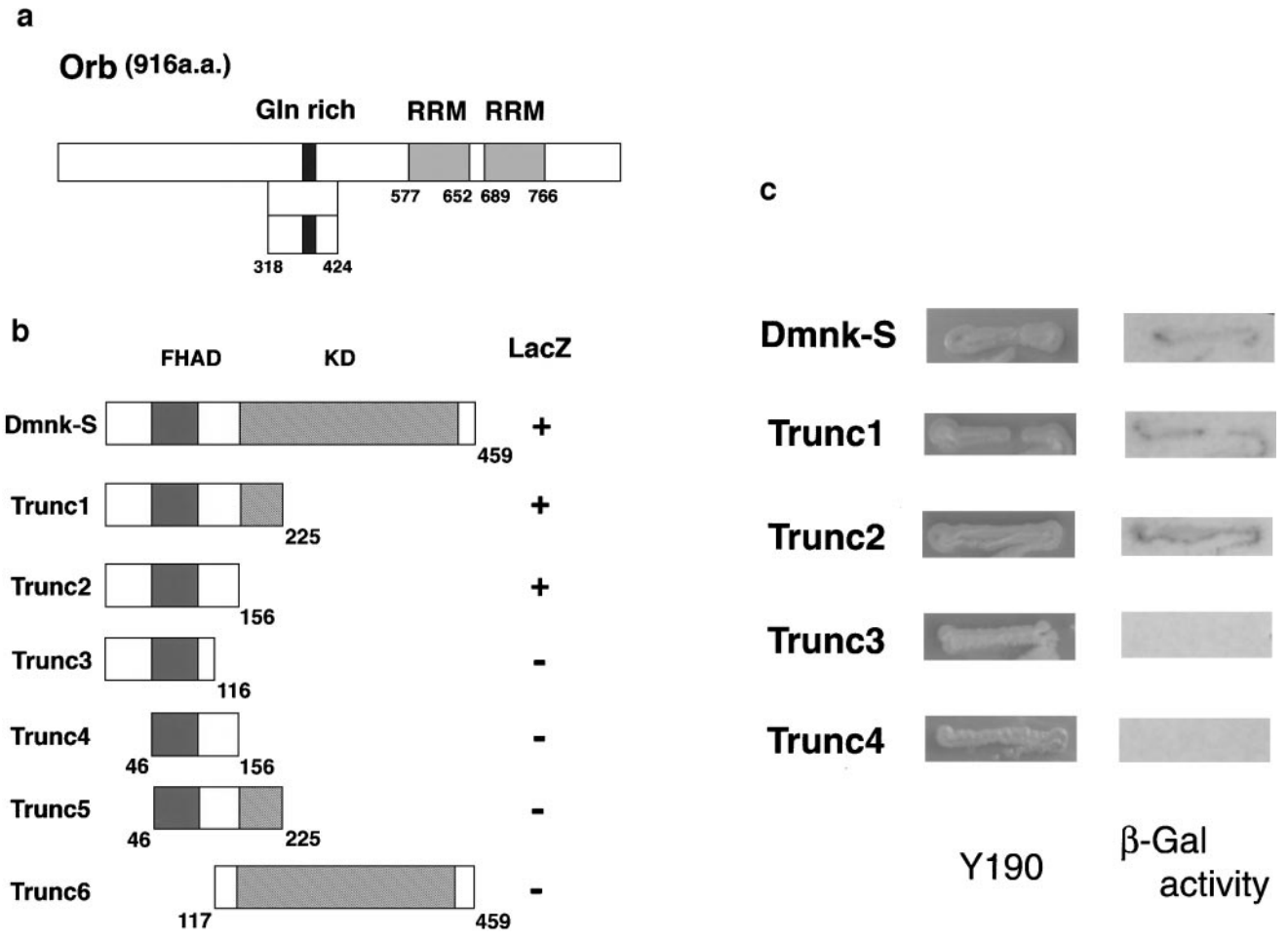

FIG. 1. Interaction of Dmnk with Orb. (a) Diagram of the Orb domain protein. The fragment of Orb protein, corresponding to 318–424 a.a., was identified as a Dmnk-S interacting domain by yeast two-hybrid screening (see Materials and Methods). (b, c) Determination of a domain(s) within Dmnk-S protein that is required for the interaction with Orb. Schematic representation of Dmnk deletion mutants utilized in this study is shown in B (Trunc 1–6). Series of constructs, encoding GAL4-DNA binding domain (GAL4BD) fused to the wild-type or mutant Dmnk-S were transfected to the yeast Y190 strain with the GAL4-DNA activating domain (GAL4AD)-Orb fusion construct, respectively. Interaction of Orb with the respective Dmnk-S mutants were analyzed by measuring LacZ expression using the β-galactosidase assay. RRM, RNA recognition motif; FHAD, forkhead-associated domain; KD, kinase domain.

regions that exhibit a high degree of similarity to the canonical RNA recognition motif (RRM) found in RNA-binding proteins (22). Genetic analyses have revealed that *orb* gene plays crucial roles in posterior determination during oogenesis, and in establishment of dorsoventral axis of embryo (23, 24). It has also been shown that, during oogenesis, Orb protein regulates the localization and translation of several maternal mRNAs, including *grk* and *osk* mRNAs that encode a dorsal determinant and a posterior organizing factor, respectively (23, 25, 26).

In the present study, we employed yeast two-hybrid screening to identify a possible binding partner(s) of Dmnk protein. We report here that Orb protein interacts physically with Dmnk. This is the first example of molecular interaction between RNA-binding protein and protein kinase that are encoded by *Drosophila* maternal genes. We also show that Orb is colocalized with Dmnk-S, but not Dmnk-L in the nuclei of culture cells, depending on its kinase activity. The functional significance of this interaction during *Drosophila* oogenesis is discussed.

MATERIALS AND METHODS

Cells and antibodies. 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS). COS-7 cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Sigma) supplemented with 10% (v/v) FBS. Yeast strain Y190 were cultured in YPD medium, containing 2% (w/v) glucose, 2% (w/v) Bacto-peptone (Difco), 1% (w/v) Bacto-yeast extract (Difco). Mouse monoclonal antibody 12CA5 (Roche) recognizes the peptide sequence (YPYDVPDYA) derived from the human influenza hemagglutinin (HA) protein (27) and mouse monoclonal antibody anti-Flag (M2, Sigma) recognizes Flag peptide sequence (DYKDDDDK).

Yeast two-hybrid screening system. The bait plasmid, pAS2-1-*Dmnk-S*, was constructed by inserting the kinase-dead version of

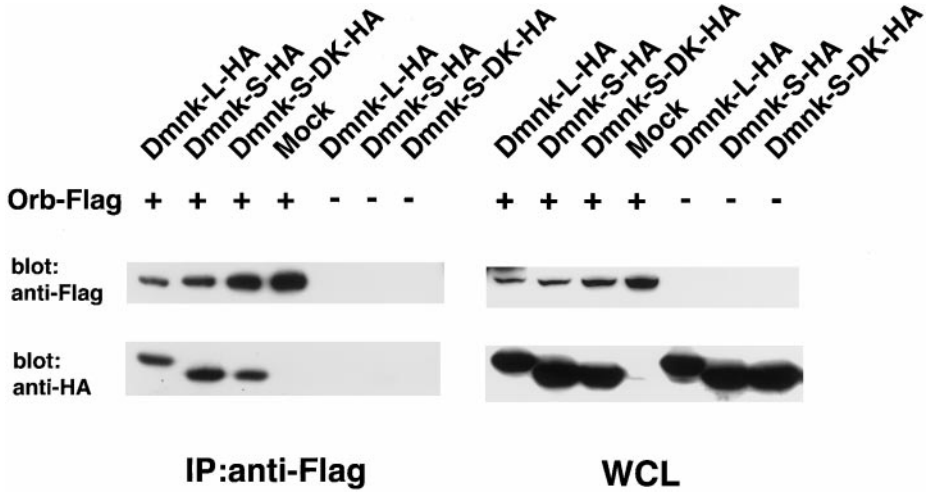

FIG. 2. Association of Dmnk with Orb in mammalian cells. 293T cells were transfected with the indicated plasmids. Whole cell lysates (WCL) or anti-Flag immunoprecipitates of WCL prepared from the respective transfectants were subjected to SDS–PAGE, blotted with anti-Flag (upper panel) and anti-HA (lower panel) monoclonal antibodies, respectively.

Dmnk-S cDNA (8) in-frame at the *NcoI* site of pAS2-1 (CLONTECH). A series of truncated *Dmnk-S* cDNAs were prepared by polymerase chain reaction (PCR) with appropriate primers by using the full-length *Dmnk-S* cDNA as a template. Yeast cells (strain Y190) containing the bait plasmid pAS2-1-*Dmnk-S* were transformed with a *Drosophila* adalt MATCHMAKER cDNA library (CLONTECH) for two-hybrid screening, using the lithium acetate method (28). Approximately 4.4×10^6 transformants were selected for their ability to grow on SD plates lacking histidine, leucine and tryptophan. The colonies grown on SD plates were subsequently analyzed for β-galactosidase activity by filter assay, and 29 β-galactosidase-positive clones were obtained. Plasmids were recovered, and transformed into *E. coli* HB101 cells by electroporation, and the cells were cultured on LB plates containing 100 μg/ml ampicillin. Library plasmids were recovered from ampicillin-resistant clones and transformed again into Y190 containing pAS2-1-*Dmnk-S*. The clones showing the $LacZ^+$ phenotypes were sequenced.

Immunoprecipitation and immunoblotting analyses. Expression vectors encoding the HA-tagged Dmnk proteins (pEF-*HA-Dmnk-S*, pEF-*HA-Dmnk-S-DK* and pEF-*HA-Dmnk-L*) were constructed as described previously (8). cDNA fragments encoding *orb* gene were amplified from *Drosophila* adult derived total RNA, by RT-PCR (8), and expression vector encoding the Flag-tagged Orb protein (pcDNA-*Orb-Flag*) was constructed. The transient cDNA transfection into 293T cells was performed using the calcium phosphate method as described previously (29). 48 h after transfection, cells were washed gently with PBS, and were solubilized with lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% (v/v) Nonidet P-40, 1 mM *p*-amidinophenylmethanesulfonyl fluoride hydrochloride (*p*-APMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin] for 30 min at 4°C. After the removal of insoluble materials by centrifugation, the precleard supernatants were immunoprecipitated with anti-Flag antibody-coupled protein A–Sepharose (Amersham Pharmacia Biotech) for 2 h at 4°C. The immunoprecipitates were washed five times with lysis buffer, and proteins were eluted by the addition of 1× Laemmli buffer. Samples were separated by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane filters. After blocking with TBST [1× TBS, 0.1% (v/v) Tween 20] containing 5% (w/v) milk, the membrane filters were incubated with appropriate antibodies for 1 h at room temperature. The filters were washed with TBST, and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (BioRad) for 30 min at room temperature. After the washes, the immunoreactive proteins were visualized by using a chemiluminescence reagent (NEN).

Immunostaining analysis. The transient cDNA transfection into COS-7 cells was performed using the SuperFect Tranfection Reagent kit (QIAGEN) following the procedure recommended by the manufacturer. Immunocytochemical staining was performed by the avidin–biotin–HRP complex (ABC-HRP) method, using the VECTASTAIN Elite ABC kit system (Vector Labs). 48 h after transfection, cells were washed gently with 1× PBS, fixed by 3.7% (v/v) formaldehyde solution for 15 min at room temperature. Subsequently, cells were washed with PBS, blocked and permeabilized with PBS containing 10% (v/v) FBS and 0.1% (v/v) Triton X-100, for 15 min at room temperature. Cells were incubated with anti-Flag (M2) or anti-HA (12CA5) monoclonal antibodies (1 μg/ml) for 30 min at room temperature. After rinsing with PBS, the cells were incubated with biotinylated anti-mouse IgG for 30 min at 37°C, washed with PBS, and then treated with the ABC reagent (avidin–biotin–HRP complex) for 30 min at room temperature. Finally, the enzymatic disclosing procedure was performed following the manufacturer's recommended protocol.

RESULTS AND DISCUSSION

In an attempt to identify a protein which interacts with Dmnk both physically and functionally, we performed yeast two-hybrid screening using the kinase-dead mutant version of Dmnk-S as a bait. 29 independent positive clones were obtained from 4.4×10^6 transformants. Among them, one clone was found to encode a part of *Drosophila* Orb protein including glutamine-rich region (Fig. 1a). To determine the Orb-binding domain within Dmnk, we examined possible association of Orb [318–424 amino acids (a.a.)] with various C-terminal or N-terminal truncated mutants of Dmnk-S by yeast two-hybrid screens (Fig. 1a). Among the C-terminal truncated Dmnk-S mutants, Trunc 3 (1–116 a.a.) but not Trunc 1 (1–225 a.a.) and Trunc 2 (1–156 a.a.), failed to associate with Orb (Figs. 1b and

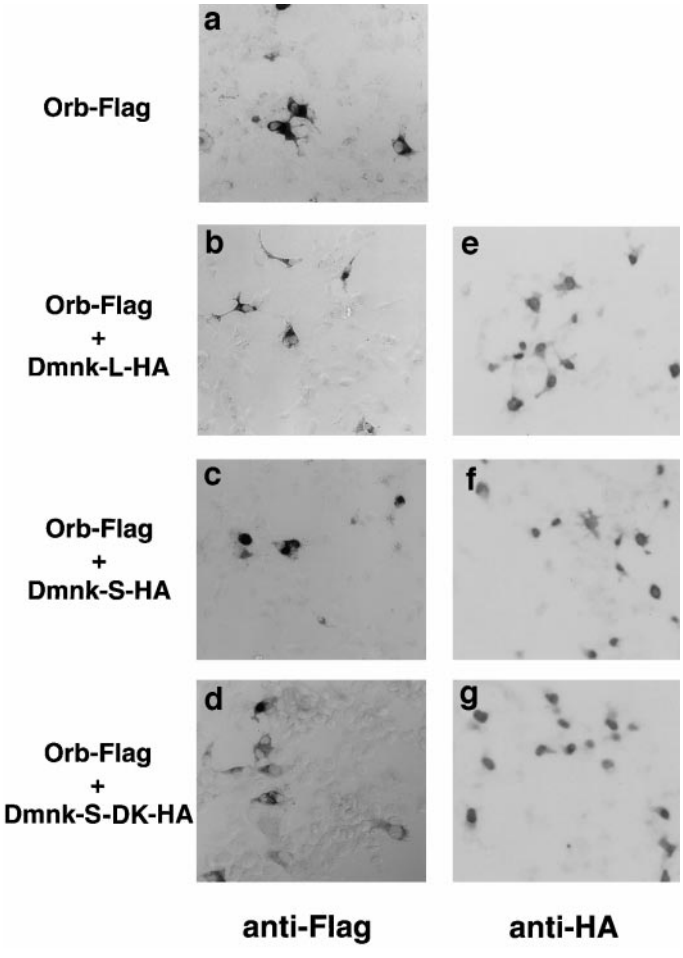

FIG. 3. Effect of different forms of Dmnk on the subcellular distribution of Orb. Series of HA-tagged Dmnks, Dmnk-L-HA, Dmnk-S-HA, or Dmnk-S-DK-HA along with Orb were expressed transiently in COS-7 cells. In some experiments, Orb alone was expressed in COS-7 cells as a control. 48 h after transfection, cells were fixed and labeled with the anti-Flag (a–d) and anti-HA (e–g) monoclonal antibodies, to determine the subcellular distribution of Orb and Dmnks, respectively. As shown, in the presence of Dmnk-S, Orb protein was localized predominantly in the nucleus (c).

1c). In addition, none of the N-terminal truncated mutations could associate with Orb (Figs. 1b and 1c). The results indicate that the N-terminal region of Dmnk-S is required for the interaction between Dmnk-S and Orb.

We next examined the association of Dmnk with Orb in mammalian cells by expressing these proteins ectopically. Flag-tagged Orb (Orb-Flag) and/or HA-tagged Dmnk-S (Dmnk-S-HA), Dmnk-L (Dmnk-L-HA), and kinase-dead mutant form of Dmnk-S (Dmnk-S-DK-HA) were transiently expressed in 293T cells. Expression of each protein in the transfected cells was assessed by anti-Flag or anti-HA immunoblots of whole-cell lysates (WCL) (Fig. 2). As shown in Fig. 2, Dmnk-S-HA, -L-HA, and -S-DK-HA were coimmunoprecipitated with Orb-Flag, indicating that Dmnk-S and Dmnk-L can associate with Orb *in vivo,* and that kinase activity of Dmnk(-S) is dispensable for this molecular interaction.

In order to pursue the possible biological significance of this molecular interaction between Dmnk and Orb, we examined the intracellular localization of Orb in relation to Dmnk in mammalian cells. To this end, Dmnk-S-HA, Dmnk-L-HA, or Dmnk-S-DK-HA along with Orb-Flag were expressed in COS-7 cells, and the subcellular localization of the respective molecules was determined by immunohistochemical analyses using monoclonal antibodies that recognize Flag and HA epitopes, respectively. As shown in Fig. 3, in the absence of Dmnk, anti-Flag signals, reflecting the localization of Orb protein, was observed in the cytoplasm, but not in the nucleus. In the presence of Dmnk-S, Orb protein was localized mainly in the nucleus, yet slightly in cytoplasm. In contrast, in the presence of Dmnk-L or Dmnk-S-DK, a mutant form of Dmnk-S lacking its intrinsic kinase activity, Orb protein was localized exclusively in the cytoplasm. In each experiment, either Dmnk-L, Dmnk-S or Dmnk-S-DK was detected mainly in the cytoplasm. These results indicate that Dmnk-S and Dmnk-L exhibit differential functions in regulating the localization of Orb protein, and that the kinase activity of Dmnk-S is required for the nuclear distribution of Orb protein.

Genetic and molecular studies demonstrate that a series of maternal genes are required at various stages of *Drosophila* oogenesis and embryogenesis. Both *Dmnk* and *orb* are maternal genes and exhibit similar expression patterns during oogenesis and embryogenesis (8, 22). In the present study, we identified Orb as an interacting protein of Dmnk by yeast two-hybrid screening and confirmed their association *in vivo* by coimmunoprecipitation assay. This is the first example of molecular interaction between RNA-binding protein and protein kinase that are encoded by *Drosophila* maternal genes. Orb plays multiple roles in oogenesis, including cystocyte formation, oocyte differentiation, posterior determination and dorsoventral axis formation (23, 24). Furthermore, it has recently been shown that Orb regulates meiosis during oogenesis (30). Although the function of Dmnk is largely unknown, the association of Dmnk with Orb suggests that Dmnk may participate in some if not all of these processes during oogenesis. Consistent with this idea, it has recently been shown that the *C. elegans* homolog of *Dmnk* plays essential roles in meiosis (11, 12, 19).

As reported previously, there are two forms of Dmnk, Dmnk-S and Dmnk-L, arising from the developmentally regulated alternative splicing of the transcript (8). The temporal expression patterns of these two transcripts during oogenesis and embryogenesis are obviously different, but their functional difference remains unclear. In this study, we showed that Dmnk-S, but not Dmnk-L, can mediate the nuclear distribution

of Orb protein in mammalian cells (Fig. 3), suggesting that these two forms may be functionally different during *Drosophila* development. In addition, it was found that Dmnk-S-mediated nuclear distribution of Orb depends on its kinase activity (Fig. 3). Thus, it can be assumed that Dmnk-S regulates subcellular localization of Orb via protein phosphorylation. Further study will be required to clarify the functional role(s) of Dmnk in cooperation with Orb in *Drosophila* oogenesis and embryogenesis.

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