Wengen, a Member of the Drosophila Tumor Necrosis Factor Receptor Superfamily, Is Required for Eiger Signaling*

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Hirosi Kanda†‡§, Tatsuaki Igaki†‡§,
Hirotaka Kanuka†‡**, Takeshi Yagi‡, and Masayuki Miura†‡‡

* The abbreviations used are: TNFR, tumor necrosis factor receptor; TNF, tumor necrosis factor; JNK, c-Jun NH2-terminal kinase; HA, hemagglutinin; RT, reverse transcription; mAb, monoclonal antibody; GFP, green fluorescent protein; CARD, caspase recruitment domain.

** Research fellow of the Japan Society for the Promotion of Science.

†‡ Research fellow of the Special Postdoctoral Researchers Program, RIKEN.

‡‡ ¶ Research fellow of the Japan Society for the Promotion of Science.

From the \§Laboratory for Cell Recovery Mechanisms, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, laboratories for Integrated Biology, Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, and \|Department of Cell Biology and Neuroscience, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

We identified Wengen, the first member of the Drosophila tumor necrosis factor receptor (TNFR) superfamily. Wengen is a type III membrane protein with conserved cysteine-rich residues (TNFR homology domain) in the extracellular domain, a hallmark of the TNFR superfamily. wengen mRNA is expressed at all stages of Drosophila development. The small-eye phenotype caused by an eye-specific overexpression of a Drosophila TNF superfamily ligand, Eiger, was dramatically suppressed by down-regulation of Wengen using RNA interference. In addition, Wengen and Eiger physically interacted with each other through their TNFR domains and were amplified by PCR and inserted into the pUAST vector (GenBank accession number for the full-length wengen cDNA, AB085747). cDNAs for a C-terminally Flag-tagged Wengen (Wengen-Flag), a Wengen without the cytoplasmic domain (WengenΔCyto-Flag), a Wengen without the TNFR homology domain (WengenΔTNFR-Flag), a C-terminally HA-tagged Wengen (Wengen-HA), an N-terminally HA-tagged Wengen (HA-Wengen), an N-terminally HA-tagged Eiger (HA-Eiger), and an Eiger with the TNFR homology domain deleted (HA-EigerΔTNF) were amplified by PCR and inserted into the pUAST vector. The expression constructs for the HA-tagged caspase recruitment domain (CARD) of DROS in flies is a powerful genetic model for studying the in vivo role of genes and their physiological regulations. Recently we identified Eiger, the first invertebrate TNF superfamily ligand, in a Drosophila misexpression screen (4). Overexpression of Eiger in the Drosophila compound eye induces cell death through the activation of the Drosophila JNK. The Eiger-induced small-eye phenotype was executed through caspase-independent pathways. It remained to be elucidated how Eiger transmits its signals to the intracellular molecules. To address this question, we have conducted a dominant modifier screen to identify downstream molecules of Eiger using eye-specific Eiger overexpressing flies (GMR>eigerW64A) and a collection of deficiency-bearing flies. Here, we describe Wengen, the first member of the Drosophila TNF superfamily, which we identified in this screen.

EXPERIMENTAL PROCEDURES

Molecular Cloning and Expression Vectors—The Drosophila EST clone SD13923 was sequenced, and the full-length wengen coding region was amplified by PCR and inserted into the pUAST vector (GenBank accession number for the full-length wengen cDNA, AB085747). cDNAs for a C-terminally Flag-tagged Wengen (Wengen-Flag), a Wengen without the cytoplasmic domain (WengenΔCyto-Flag), a Wengen without the TNFR homology domain (WengenΔTNFR-Flag), a C-terminally HA-tagged Wengen (Wengen-HA), an N-terminally HA-tagged Wengen (HA-Wengen), an N-terminally HA-tagged Eiger (HA-Eiger), and an Eiger with the TNFR homology domain deleted (HA-EigerΔTNF) were amplified by PCR and inserted into the pUAST vector. The expression constructs for the HA-tagged caspase recruitment domain (CARD) of DROS in vitro. S2 cells (5) were cultured at 26 °C and transfected with the constitutively active dominant-negative form of JNK. The Eiger-induced small-eye phenotype would be suppressed in the F1 progeny.

Cell Culture, Transfection, Immunostaining, and Preparation of Cell Lysates—Drosophila S2 cells (5) were cultured at 26 °C and transfected using Cellfectin (Invitrogen) as described previously (6). For immunostaining, an anti-HA monoclonal antibody (1:200; 12CA5, Roche Molecular Biochemicals) and a Cy5-labeled anti-mouse IgG secondary antibody (1:100; Chemicon) were used. For the detection of HA-Wengen and HA-CARD, S2 cells were transiently transfected with expression vectors together with pWAGAL4. Twenty-four hours after the transfection, cells were lysed in 1.5× SDS sample buffer containing 75 mM Tris-HCl (pH 6.8), 150 mM dithiothreitol, 3% SDS, 0.15% bromphenol blue, 9%
FIG. 1. Identification of Wengen, a member of TNFR superfamily. Light microscopy micrographs of wild-type (A); +/+; regg1G20K37+/+; GMR-GAL4/+ (B); and +Df(1)E128; regg1G20K37+/+; GMR-GAL4/+ (C) flies are shown. D, cytological map of the deficiency line, Df(1)E128/FM7c. E, amino acid sequence of Wengen. The red box indicates the cysteine-rich domain (or TNFR homology domain), and the blue box indicates the membrane-spanning region (transmembrane domain). F, schematic structures of Wengen, hXEDAR, and hTNFR1. Extracellular TNFR homology domains (red box, A1 and B2 modules; light red box, A1 and C2 modules) are conserved. TM, transmembrane domain. G, deduced amino acid sequence of the TNFR homology domain of Wengen is aligned with those of hXEDAR and hTNFR1. H–K, the expression constructs for C-terminally HA-tagged Wengen and GFP were transiently transfected into S2 cells with pWAGAL4. Twenty-four hours after transfection, the cells were immunostained with an anti-HA mAb after (H and I) or before (J and K) fixation and permeabilization. L, expression of wengen was examined by RT-PCR at various stages of Drosophila development (embryo, larvae, pupae, and adult). GAPDH (glyceraldehyde-phosphate dehydrogenase) primer set was used as a control.

glycerol, 4% 2-mercaptoethanol. For the immunoprecipitation assay, S2 cells were transiently transfected with each expression vector together with pWAGAL4. Twenty-four hours after the transfection, cells were lysed in 500 µl of lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 0.5% deoxycholic acid, and 1 mM phenylmethylsulfonyl fluoride.

Reverse Transcription PCR—Total RNA was isolated from embryonic, larval, pupal, and adult stages of Drosophila and amplified by reverse transcription (RT)-PCR as described previously (4). wengen cDNA was amplified by using the following primers: 5′-ATG CGT AGT CGG AGC AGC AGT G-3′ and 5′-GTG GTG GAT GAT GAG GAT GCA GGC GGC C-3′. The primer sequences used for detection of glyceraldehyde-phosphate dehydrogenase cDNA have been described (7). To detect the expression of wengen or GAPDH, 32 or 26 cycles of PCR were performed, respectively, and then their expression was analyzed on an agarose gel.

Western Blotting and Immunoprecipitation—For the detection of HA-Wengen and HA-CARD, an anti-HA polyclonal antibody (1:500, MBL) and a horseradish peroxidase-conjugated secondary antibody (1:1000, anti-rabbit IgG; Transduction Laboratories) were used. For the immunoprecipitation assay, cell lysates were incubated with an anti-Flag M2 affinity gel (Sigma) or a protein G-Sepharose-conjugated anti-HA 3F10 mAb (Roche) at 4 °C overnight. Samples were separated by SDS-PAGE and analyzed by immunoblot analysis with appropriate antibodies.

RESULTS

In a Drosophila dominant-modifier screen using the chromosomal deficiency lines that covered more than 70% of the genome, we obtained several lines that suppressed the small-eye phenotype caused by Eiger overexpression (GMR>elit). (4) (Fig. 1, A–C). Through the analysis of these deficiency lines, we identified a line, Df(1)E128/FM7c (8), in which the deficiency spans the coding region of a predicted gene, CG6531 (Fig. 1D). CG6531 was speculated to encode a protein with a cysteine-rich domain (TNFR homology domain), the hallmark of the TNFR superfamily (3). We named this gene wengen for a village at the foot of Mt. Eiger. Analysis of the wengen nucleotide sequence revealed an open reading frame of 343 amino acids with a predicted relative molecular mass of 40 kDa (Fig. 1E and data not shown). Alignment analysis revealed that Wengen harbored a TNFR homology domain in the extracellular region and a membrane-spanning region without signal sequence (Fig. 1, E and F). This is a characteristic of the type III membrane protein of TNFR superfamily (extracellular N terminus, intracellular C terminus, lacking a signal peptide) (1). The TNFR homology domain of Wengen had significant structural and amino acid homology with the TNFR domains of human EDAR (hXEDAR) and human TNFR1 (hTNFR1) (Fig. 1G).

The TNFR homology domains of Wengen, hXEDAR, and hTNFR1 share the topologically distinctive modules (termed A1 and B2 module, except for the TNFR homology domain of hTNFR1 (amino acids 168–195), which is composed of the A1 and C2 modules) (9). To investigate whether Wengen was indeed a type III membrane protein like the other TNFR superfamily members, we examined its subcellular localization (Fig. 1, H–K). S2 cells were transiently transfected with expression vectors for C-terminally HA-tagged Wengen (Wengen-HA) and GFP. In GFP-positive cells, which were assumed to be overexpressing Wengen-HA, Wengen-HA was detected on the surface membrane with anti-HA antibody when the S2 cells were permeabilized (Fig. 1, H and I); the signal was not detected in GFP-positive cells without permeabilization (Fig. 1, J and K). These data suggest that the Wengen C-terminal domain is indeed cytoplasmic. These results suggest that Wengen is a member of the type III TNFR superfamily. We then examined the expression of wengen in flies. RT-PCR analysis revealed that wengen mRNA was expressed at all stages of development (Fig. 1L). Its putative ligand, Eiger, is also expressed at all stages of Drosophila development (4).
To assess whether Wengen is required for Eiger to induce the small-eye phenotype, we used RNA interference (RNAi) to down-regulate the endogenous expression of Wengen. A head-to-head inverted repeat construct for \textit{wengen}, pUAS-\textit{wengen-IR}, was generated, and we examined its ability to knock down the \textit{wengen} expression (Fig. 2A). Co-transfection of pUAS-HA-\textit{wengen} together with pUAS-\textit{wengen-IR} into S2 cells dramatically reduced Wengen expression but had no effect on the expression of HA-CARD (Fig. 2B), suggesting that \textit{wengen-IR} works as a specific inhibitor of Wengen expression. To assess the biological functions of Wengen in \textit{Drosophila}, we generated transgenic flies that misexpress \textit{wengen-IR} in the developing retina. The small-eye phenotype induced by the eye-specific ectopic expression of Eiger (\textit{GMR>}\textit{eiger}\textsuperscript{regg1}, Fig. 2C) was suppressed by the coexpression of \textit{wengen-IR} (Fig. 2D). These results strongly suggest that Wengen is required as a functional transducer of Eiger signaling.

Next, we assessed the physical interactions between Wengen and Eiger using various deletion mutants (Fig. 3A). Immuno-precipitation assays revealed that full-length Wengen and Eiger physically interacted with each other (Fig. 3B, \textit{first lane}, and C, \textit{first lane}). Eiger interacted with Wengen\textit{Δcyt} but not
with WengenΔTNFR (Fig. 3B, second and third lanes). In addition, full-length Wengen could not interact with Eiger ΔTNF (Fig. 3C, second lane). These results suggest that Wengen can interact with Eiger, and this interaction is mediated through the TNFR homology domain of Wengen and the TNF homology domain of Eiger.

**DISCUSSION**

In this study, we identified the first *Drosophila* member of the TNFR superfamily, Wengen. Most of the genes for the TNFR superfamily encode type I or III membrane proteins with one or more extracellular ligand-binding domains and a cytoplasmic region that activates cell functions. In general, the extracellular domain of this family of proteins shows a relatively low level of sequence conservation, despite sharing a common fundamental structure. The cytoplasmic regions of the receptors show considerably more diversity in sequence and size than the extracellular regions. There are no common intracellular motifs found in all members of the TNFR superfamily except for some domains such as the TRAF2-binding domain ((P/S/A/T)x(Q/E)E or PXQXxD) (10), which is required for both NF-κB activation and JNK activation, or a domain of ~80 amino acids called the “death domain” (3) for caspase activation. However, the amino acid sequence of Wengen reveals that it has neither a TRAF2-binding domain nor a death domain in the cytoplasmic region, suggesting that there should be another mechanism to transduce signals.

Whereas Eiger can stimulate the JNK pathway (4), we failed to detect the stimulation of the JNK pathway in response to the overexpression of Wengen in S2 cells or the *Drosophila* compound eye (data not shown). It is possible that because the amount of ligand is limited, overexpression of Wengen was not sufficient to activate the downstream signals. It is also possible that intracellular adapter proteins, which are required for transducing signals, are not expressed or limited in Wengen-expressing cells. Otherwise, Wengen may require one or more co-receptors that transduce signals to the cytoplasm. For instance, heteromeric receptor complex is used to transduce Hedgehog signaling (11). Hedgehog binds to its receptor Patched, and then the inhibitory function of Patched against its binding partner, Smoothened, is cancelled. In this way, Hedgehog signaling is transduced into the cells. Because the heteromeric complex of receptors has never been reported to transduce TNF family signaling, it is possible that Eiger/Wengen may use the novel type of TNF signaling mechanisms. In any case, further genetic and biochemical studies of Eiger/Wengen should help to elucidate the unique signaling mechanisms that include the caspase-independent pathway triggered by Eiger.

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