The product of the *Drosophila melanogaster* odd Oz (odz)/Tenascin-major (ten-m) pair-rule gene consists of eight epidermal growth factor (EGF)-like repeats followed by a novel 1800 amino acid polypeptide stretch unique to proteins of the Odz/Ten-m family. The structure and membrane orientation of this large enigmatic protein was characterized by raising and employing antibodies directed against discrete Odz polypeptide regions. Protein-modifying reagents impermeable to the plasma membrane were used in concert with the battery of antibodies to demonstrate that Odz is a type I transmembrane protein with the vast C-terminal portion in the intracellular space, and with the EGF repeats deployed extracellularly. The polypeptide was shown to undergo multiple cleavages at discrete intracellular and extracellular sites, and its extreme C-terminus was shown to undergo either processing at a very large number of sites or programmed degradation. The polypeptide is presented at the cell surface with additional post-translational modifications, and as two subunits of previously cleaved Odz joined by cysteine disulphide bridges maintaining their association. The model derived for the Odz protein is discussed in light of other models proposed for proteins of the Odz/Ten-m family, and in terms of functional implications.

Key words: EGF-like repeat, Notch, pair-rule gene, segmentation, signal transduction.

| Abbreviations used: odz, odd Oz gene; ten-m, Tenascin-major gene; EGF, epidermal growth factor. |
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protein in the extracellular matrix [4]. On discovery of the vertebrate homologues, a type I protein form was proposed [14,17], as was a type II transmembrane protein form [9,11]. All models agree that the EGF-like repeats are deployed extracellularly, as per all precedents. On the rest of the structure, and especially the location of the large C-terminal portion of the protein, the models are contradictory. At issue are a highly hydrophobic region of the protein N-terminal to the EGF-like repeats, viewed as a transmembrane domain versus a signal-peptide domain, and a hydrophobic region C-terminal to the EGF-like repeats, viewed as a transmembrane domain or as a stretch that is insufficiently hydrophobic to act as a transmembrane-spanning region.

Given the lack of precedents for the sequence domains in question, determining the protein’s structure and deployment relative to the cell membrane empirically might prove to be one of the most important steps towards elucidating its mechanism of function. In this study we have produced and utilized a bank of seven antibodies against the Drosophila Odz protein to address the structural and functional question, determining the protein’s structure and deployment empirically, as per all precedents. On the rest of the structure, and especially the location of the large C-terminal portion of the protein, the models are contradictory. At issue are a highly hydrophobic region of the protein N-terminal to the EGF-like repeats, viewed as a transmembrane domain versus a signal-peptide domain, and a hydrophobic region C-terminal to the EGF-like repeats, viewed as a transmembrane domain or as a stretch that is insufficiently hydrophobic to act as a transmembrane-spanning region.

Non-denaturing purification of 6×His-Odz cytoplasmic and periplasmic proteins

Crude extracts A (cytosolic soluble proteins, see above) were added to 50% slurry of Ni²⁺-nitriloacetic acid–agarose resin (Qiagen) equilibrated previously in sonication buffer. The resin and extract slurry were stirred on ice for 60 min, loaded into a 1.6 cm-diameter column, and washed with sonication buffer (flow rate, 0.5 ml/min) until the flow-through Aₐₘ was less than 0.01. Another wash was carried out with wash buffer (50 mM sodium phosphate/300 mM NaCl/10% glycerol, pH 6.0) until the flow-through Aₐₘ was less than 0.01. Proteins were eluted with a gradient of 0–0.5 M imidazole in wash buffer. The resulting fractions were dialysed against two consecutive 1 litre changes of PBS.

Crude extracts C (periplasmic extracts) were dialysed against three 1 litre changes of sonication buffer before mixing the extracts with Ni²⁺-nitriloacetic acid–agarose resin as described above for cytosolic soluble proteins.

Production and purification of antibodies against 6×His-tagged fusion proteins

Odz fusion protein containing fractions eluted from Ni²⁺-nitriloacetic acid–agarose resin columns were identified by SDS/PAGE and Coomassie Brilliant Blue staining. Purified protein (0.5–1.5 mg) was used either directly for immunizations (non-denatured) or for immunization after purification by excision after PAGE (denatured). These fractions were injected into guinea pigs and rats for producing polyclonal antibodies, named according to the immunizing fusion proteins: 700EC, 299EGF, 166ATM, 334Y1, 489Y2, 1329Y3/4 and 912YS.

Once antisera were raised, they were affinity-purified on columns containing Odz fusion-protein fragments. Briefly, 0.5–1.0 mg of purified 6×His-Odz proteins were dialysed against 10 mM Mes, pH 6.0, and coupled to 1 ml of N-hydroxysuccinimide Affigel-10 columns (Bio-Rad) according to the manufacturer’s protocol. The remaining activated groups were blocked with 0.1 M ethanolamine, pH 8.0. The affinity matrix was washed with 10 ml vol. of 10 mM Tris, pH 7.5, followed by 10 mM Tris, pH 7.5/500 mM NaCl, and then by 10 mM glycine, pH 2.5. Subsequently, washes were carried out with 10 mM Tris, pH 8.8, until the pH was neutralized, followed by 10 vol. of fresh 0.1 M triethylamine, pH 11.5, and 10 mM Tris, pH 7.5, for neutralizing/stabilizing the pH again. The affinity matrix for each protein was incubated with 5 ml of anti-Odz antiserum and washed with 10 mM Tris, pH 7.5, and 10 mM Tris, pH 7.5/500 mM NaCl. Antibodies were eluted with 100 mM glycine, pH 2.5, and neutralized immediately with a one-tenth vol. of 1 M Tris, pH 8.8. Alternatively, antibodies were eluted with 100 mM triethylamine, pH 11.5, and neutralized immediately with a one-tenth vol. of 1 M Tris, pH 7.0. All purified antibodies were dialysed against two 2 litre changes of PBS. The monoclonal antibody mAb20, which specifically identifies Odz in Western-blot analyses, was raised as described previously [3].

Western-blot analysis

Samples of protein (5–30 μg) derived from embryos, pupae, adults and S2-Schneider cells were prepared rapidly as 1:2 dilutions with 2× loading buffer (50 mM Tris, pH 6.8, 1 mM EDTA, 4% SDS, 25% glycerol, 0.2% Bromophenol Blue and...
up to 6 % 2-mercaptoethanol) in order to prevent post-harvest degradation of protein. Extract preparations were sampled and compared with equivalent small samples rapidly extracted directly into reducing sample buffers with 1 % SDS, which were boiled immediately or treated at 95 °C, as well as other regimens that prevent post-extraction degradation. The comparisons insured that the extraction methods did not introduce post-harvest degradation. When non-reducing conditions were used for PAGE, 2-mercaptoethanol was omitted from sample buffer. Western blotting was performed by using nitrocellulose membranes (NC-45; Schleicher & Schuell) in Tris/glycine buffer containing 20 % methanol for 2 h at 40 V in a Bio-Rad mini-gel transfer system. The membranes were blocked with 10 % low-fat milk in PBS/0.05 % Tween 20, incubated with polyclonal antibodies against Odz (dilutions, 1:300–1:500) or monoclonal antibody mAb20 (dilution, 1:2000) and developed with horseradish peroxidase-conjugated secondary antibody (dilutions, 1:5000–1:12000) using the ECL detection system (Amersham Bioscience).

In order to compare antibodies on the same membranes, stripping of the nitrocellulose membranes was performed up to three times with the same membrane. Stripping was carried out in 62.5 mM Tris, pH 6.7, 2 % SDS and 100 mM 2-mercaptoethanol followed by extensive washes with PBS/0.1 % Tween 20. In order to ensure complete removal of the antibodies, the membrane was observed using the ECL detection system before reuse. These membranes were kept in PBS at 4 °C between immunodetection rounds.

**Biotinylation of surface-protein determinants**

S2-Schneider cells were grown in 10 cm plastic tissue-culture plates to ≈ 60 % confluence. Three plates were used per sample (with or without biotin samples). Cells were washed four times with cold PBS-CMG (PBS/0.1 M CaCl₂/1 M MgCl₂/1 % glucose, pH ≈ 8.0). Fresh PBS-CMG (2 ml) with 0.5 mg/ml sulpho-NHS-biotin (Pierce) was added to each plate, then incubated at 4 °C for 20 min with mild shaking (as described in [26]). As a biotin-free control, PBS-CMG was added to parallel plates and processed identically. The incubation (or control) solution was replaced with cold Schneider medium (Invitrogen-Gibco, Carsbad, CA, U.S.A.) without serum to react with excess biotin, and was incubated at 4 °C for 15 min. Cells were washed four times in cold PBS-CMG and then lysed in 0.5 ml of lysis buffer/sample (150 mM NaCl/10 % glycerol/1 mM EGTA/20 mM Hepes, pH 7.5/1.5 mM MgCl₂/1 % Triton-X-100). After addition of SDS to 0.2 %, 400 µl of each sample (with or without biotin) were used for precipitation with 20 µl of immobilized avidin (Pierce). Samples were incubated overnight at 4 °C and washed twice with 0.5 ml of lysis buffer plus protease inhibitors (Sigma): 1 mM PMSF, 0.5 mM benzamidine, 1 µg/ml aprotinin and 1 µg/ml leupeptin. Samples were resuspended in 2 × sample buffer, boiled and subjected to SDS/PAGE and Western-blot analysis.

**Digestion of surface-protein determinants with trypsin and proteinase K**

S2-Schneider cells were grown in 10 cm plastic tissue-culture plates to ≈ 60 % confluence. Cells were washed three times with cold PBS. An aliquot of the cells was incubated with 0.25 % trypsin, in PBS, at room temperature (22 °C) for 0–90 min and another aliquot was incubated with 0.25 % trypsin plus 0.1 mg/ml proteinase K for 60 min. Microscopic monitoring of cells’ vitality was tracked with Trypan Blue throughout all time points. The digestion process was stopped by adding Schneider medium supplied with 10 % fetal calf serum. Cells were then harvested and the pellets were washed three times with PBS. Samples were resuspended directly into 2 × sample buffer, boiled and subjected to SDS/PAGE.

**Immunoprecipitation of Odz from Schnieder cells**

Cells were solubilized in ice-cold lysis buffer supplied with 1 mM PMSF, 0.5 mM benzamidine, 1 µg/ml aprotinin and 1 µg/ml leupeptin. The extract was centrifuged for 5 min at 12000 g. Extract usually included 1 mg/ml protein, as measured in the Bio-Rad Bradford protein assay. Immunoprecipitation of Schneider cells was carried out using 5 µl of mAb20 or 4 µl of the purified polyclonal antibodies 489Y2 or 700EC. The antibodies were incubated with 20 µl of packed Protein A beads for 1 h at 4 °C, followed by three washes with lysis buffer. Protein extract (200 µg) was added and incubated for 16 h at 4 °C, followed by two washes with lysis buffer and a last wash with 20 mM Hepes. The immune complexes were harvested, resuspended in 2 × loading buffer and analysed by SDS/PAGE.

**Heat shock of Odz transgenic flies**

Activation of the complete odz gene coding region or partial odz (nucleotides 1–4260) by means of the heterologous Hsp70 promoter was carried out for several families (independent inserts of given transgene constructs) of trangenes. Adult flies of the age 3–8 days post-eclosion were placed in a 37 ºC bath for 15 min, and then homogenized 2 h later in 2 × sample buffer, boiled and subjected to SDS/PAGE.

**X-gal staining of embryos**

X-gal staining was carried out on Canton-S, Samarkand and Df(3L)odz-AL1/ftz-lacZ TM3Sb offspring embryos. The embryos were stained much as described previously [27]. The embryos were washed twice with PBS, then according to their staining or lack thereof, were separated as white (odz homozygotes) and blue [Df(3L)odz-AL1/ftz-lacZ TM3Sb-(odz⁺) or ftz-lacZ TM3Sb-(odz⁺) homozygotes] embryos under a binocular dissecting microscope. Equal amounts of white/blue embryos were homogenized directly into 2 × sample buffer, boiled and subjected to SDS/PAGE, followed by staining with Coomassie Brilliant Blue or processing in Western blots with appropriate antibody probes.

**RESULTS**

**Odz fusion proteins prepared to use as antigens**

The Drosophila odz/ten-m gene is transcribed as 10.5–11.5 kb messages [4], which encode a 2731-amino acid protein [3,5]. The initial characterization of the protein was carried out using monoclonal antibody mAb20, identifying a polypeptide corresponding to full-length Odz in Western blots. However, the predominant polypeptides detected in these analyses were of smaller reproducible size, suggesting a programmed cleavage of the Odz protein. In order to further our understanding of the structure of the Odz protein, it was imperative to create tools to recognize the various cleaved Odz species. Accordingly, we expressed polypeptides encoded by seven different portions of the odz coding region to serve as antigens to raise a panel of seven monoclonal antibodies. For each of the seven regions chosen as antigens to be expressed. Each of the seven partial Odz polypeptides was expressed in bacteria as a fusion protein with a 6 × His N-terminal tag. Fusion proteins were prepared in order to directly antibodies against the region.
that lies N-terminal of Odz EGF repeats (700EC); the EGF repeats (299EGF); the region just C-terminal to the EGF repeats which is highly conserved in Odz of all species (166ATM) and against four consecutive C-terminal regions each carrying at least one tyrosine-phosphorylation consensus site (334Y1, 489Y2, 1329Y3/4 and 912Y5). The region containing the epitope of mAb20, described above, appears on Figure 1(A).

Each expressed fusion protein differentially accumulated in the bacterial cytoplasm, in the periplasmic space or in insoluble compartments in bacteria (Figure 1B). The fusion proteins were collected and purified from the appropriate fractions, and were then used as non-denatured or denatured antigens (see the Experimental section). Once purified on nickel-affinity columns and PAGE, the bacterial Odz fusion proteins were of the expected sizes (Figure 1C), except for the construct 299EGF, which ran at a size equalling that of a dimer (28 kDa). These bacterial extracts of the 299EGF construct, which encodes EGF repeats 3–8 of the Odz protein, were not run here under strong reducing conditions (Figure 1C), but the dimer size converts to monomer mobility under stringent reducing conditions (results not shown). The capacity of this portion of the Odz protein to self-dimerize via cysteine S-S linkages is an indication that the EGF-like repeat domains have an intrinsic ability to form homodimers. This is supported by predicted size-coassociated forms in Drosophila cells under non-reducing conditions (below), and in work on mouse Ten-m2 (Odz2) [11].

Raising antibodies against discrete Odz protein regions and verifying their specificity

Each of the Odz fusion proteins was used to raise antisera in guinea-pigs, rats or both. Verification that the antiseras raised contained specific anti-Odz reactivity was complicated by the fact that the Odz protein was detected predominantly as a multiply cleaved polypeptide. Consequently, Western-blot analysis for each antiserum was expected to primarily show Odz polypeptides of non-predictable sizes. Therefore, discriminating between a serum’s Odz-specific Western-blot reactivity versus potentially artifactual Western-blot signals could not be based on expected band mobility. We instead had to employ assays to stringently identify true Odz Western-blot bands of non-predicted sizes. To do this, we prepared two pairs of genetically distinct sources of extracts which would provide conclusive Odz+ and Odz- controls.

The first pair was a system in which embryos were genetically marked: those with third chromosomes carrying both a wild-type odz gene and a ‘blue’ (β-galactosidase-encoding) marker and those with third chromosomes carrying odz+ mutant alleles with no Lac-Z marker. The embryos carrying at least one odz+ chromosome were distinguishable from their odz homozygous siblings by their blue colour when incubated with X-gal. Extracts of populations of hand-picked X-gal-stained odz- (white) versus

Figure 1  Expression of discrete Odz protein regions used as immunizing antigens

(A) odz gene coding-region stretches selected for raising polyclonal antibodies. An alignment appears between the significant protein features (Odz protein, right); blocks of the coding region chosen to be expressed for antibody preparation (expressed subcloned domains, middle) and the restriction sites used for subcloning from cdNA (left); restriction enzymes appearing in parentheses are those cases where restriction sites from the polylinker of the vector abutting the restriction sites used for subcloning from cDNA, left: restriction enzymes appearing in parentheses are those cases where restriction sites from the polylinker of the vector abutting the insert were used). The significant features of the Odz protein sequence are eight EGF-like repeats, bounded by two hydrophobic stretches (putative signal peptide and putative transmembrane). Five tyrosine-phosphorylation consensus sites (Y-P) and four glycosaminoglycan consensus attachment sites (horizontal bars) also occur. The sequence blocks chosen to be expressed in 6 x His expression vectors are named according to the number of base pairs of coding region included in the construct and an abbreviation of some feature of the coding area. As such, they are: 700EC, 700 bp extracellular; 299EGF, EGF-like repeats; 166ATM, after transmembrane; 334Y1, 489Y2, 1329Y3/4, 912Y5, first–fifth tyrosine-phosphorylation consensus sites. The equivalent scales for 300 bp and 100 amino acids are shown. The range containing the epitopes of the mAb20 monoclonal antibody is shown by a bracket labelled MAb20. (B) Bacterial intracellular distribution of the expressed 6 x His-Odz-fragment fusion proteins. Coomassie Brilliant Blue-stained SDS/PAGE; for details see the Experimental section. Each of the expressed Odz-fragment polypeptides preferentially accumulated in fractions A (cytoplasm), B (insoluble material) or C (periplasmic space) of the bacterial expression host. M, protein molecular-mass marker. Arrows indicate mobility of expressed fusion proteins. (C) Examples of purified Odz-fragment fusion proteins used for raising polyclonal antibodies: Coomassie Brilliant Blue-stained PAGE.
odz+ (blue) embryos were run side by side to discern true Odz Western-blot bands (Figure 2A). Despite identical overall protein content, the odz- extracts displayed no Western-blot reactivity with Odz antibody.

The second pair of genetically distinct sources of extracts provided the basis of an independent assay to identify true Odz bands which reacted with antisera. This approach utilized induced expression of a full-length form of the odz transgene (or a truncated form in alternative experiments), under the control of a heat-shock promoter (Hsp70). Expression of the transgene was induced at developmental stages when Odz was expressed at very low endogenous levels. Extracts from heat-shocked adult Hsp70-odz transgenic flies were compared with extracts from noninduced flies and extracts from wild-type flies (Figure 2B). Only very weak, and very low-molecular-mass, immunoreactive Odz material was detected in control flies, providing a low background level against which to observe the inducible and trackable Odz expression.

An example of proof that a given polyclonal antibody recognized specific Odz bands of previously unknown sizes can be seen in Figure 2(C). Here too, testing of an antibody-purification regimen is demonstrated. By comparing the two left-hand lanes (before affinity-purification of the 489Y2 antibody) and the two right-hand lanes (after affinity-purification) in Figure 2(C), bona fide Odz polypeptide bands are distinguishable from artifactual Western-blot signals. Similar purification regimens and rigorous tests were performed for all of the antibodies raised, and the results that follow are based on the use of antibodies with their specificity proven by one or both of the methods described here.

All anti-Odz antibodies primarily recognize high-mobility Odz forms, predicting specific cleavage sites

We have seen that very little full-length Odz protein is observed in Western blots using any anti-Odz antibody. Therefore, great lengths were taken to ensure that no post-harvest degradation or cleavage of Odz occurred, and that the species of Odz visualized truly reflected the state of the protein before extraction from tissue-culture cells or flies (see the Experimental section). By cataloging the sizes of Odz fragments recognized by antibodies directed against each discrete region of the protein, a model of the cleavage sites utilized in its processing could be constructed. The antibodies 166ATM and 489Y2, directed against central portions of the coding region, detect a small amount of very large protein (300 kDa) whose size is consistent with full-length Odz. However, these two antibodies primarily recognize a common subset of smaller polypeptides (Figure 3, top panel). Detection of 65 and 75 kDa Odz fragment species was common to the two antibodies in both fly embryos and Schneider tissue-culture cells. These two different fragments were generated either from an additional cleavage of the 75 kDa fragment to 65 kDa, or by other differential post-translational events leading to the two alternative mobilities. In contrast, only antibody 166ATM, with determinants N-terminal to those of 489Y2 (see Figure 1A), detected a 105 kDa species and 45 kDa species, implying that these two Odz fragments contain regions of the protein encoded by more N-terminal-corresponding portions of the gene. These and other bands’ differential preponderance in embryos, Schneider cells and pupae (results not shown) indicates that a subset of the cleavage or other post-translational events vary in frequency at different developmental stages.

The predominant fragment sizes and antigen-determinant boundaries were used to deduce approximate cleavage sites of the Odz polypeptide, as shown in Figure 3 (bottom panel). This representation is an integration of the data from 166ATM, 489Y2, 334Y1 (results not shown) and other antibody results described below. The placement of cleavages in the model conform to the 65 kDa (75 kDa) fragment (resulting from cleavage at sites c and e in Figure 3, bottom panel). 166ATM, but not 489Y2, reacted with the 45 kDa fragment (sites b and d in...
Figure 3  Fragments recognized by antibodies directed against specific Odz regions

Top panel: processed Odz protein fragments recognized by polyclonal antibodies 166ATM and 489Y2. Protein extracts of Schneider cells (S2) or embryos (E) were subjected to PAGE. Western-blot analysis was then carried out using affinity-purified (see the Experimental section) polyclonal antibodies 166ATM (166) or 489Y2 (489), pre-screened for Odz specificity. Bottom panel: schematic model of Odz cleavage sites based on Western blots using the panel of polyclonal antibodies. The relative positions of the areas of the Odz coding region utilized to raise polyclonal antibodies appear on the left, along with the positions of tyrosine-phosphorylation consensus sites (Y-P) for orientation. Cleavage sites a–e are placed approximately on the basis of the antigens’ positions and Western-blot data.

Figure 4  Fragments recognized by antibodies directed against additional Odz regions

A cDNA-based transgene expressing full-length Odz under heat-shock promoter control was heat shocked (h.s.) for 15 min, followed by a 2 h recovery at room temperature, in adults, which have very low levels of endogenous Odz expression. Protein extracts of flies that underwent heat shock (+) or not (−) were subjected to PAGE followed by Western-blot analysis employing anti-Odz antibodies. Several independent families of flies carrying the Odz full-length transgene in a yellow white genetic background (YW/full cons.) were assayed, as well as yellow white flies without the transgene (YW). (A) Odz fragment sizes recognized by polyclonal antibody 700EC. (B) Odz fragment sizes recognized by polyclonal antibody 299EGF; in (A) and (B), Schneider S2 cell protein extracts were run in parallel (S2). (C) Odz fragment sizes recognized by polyclonal antibody 166ATM; pupal-stage protein extracts were run in parallel (P).

Antibodies generated against more N-terminal portions of the Odz polypeptide, 700EC and 299EGF, likewise share between them recognition of products of an integrated series of cleavage sites (Figure 4A for 700EC, Figure 4B for 299EGF, and see also Figure 3, bottom panel). These two antibodies detected 300 kDa bands indicative of full-length Odz, but recognized more prominent Odz fragments of lengths 70 and 105 kDa. Antibody 700EC also recognized a band of 40 kDa, a product of internal cleavage of the 70 kDa protein which is not reactive to 299EGF. The 70/40 kDa fragments are the most N-terminal fragments mapped in the cleavage sequence (cleavage sites a and b in Figure 3, bottom panel). The less prominent 105 kDa band is mapped to the same N-terminal cleavage site as that of the 70/40 kDa species, is fully inclusive of them, but extends more C-terminally (cleavages a and c in Figure 3, bottom panel).

To integrate the cleavage sites of the N-terminal portion and the middle portion of the protein, a comparison was made between Western-blot bands common to the two pairs of antibodies (700EC and 299EGF versus 166ATM and 489Y2). By reacting antibodies 299EGF and 166ATM serially to the same filters, it can be seen that they detected 45 kDa bands with precisely the same mobility. Mapping is consistent with these two antibodies recognizing the same 45 kDa fragment (Figures 4B and 4C, cleaved at points b and d in Figure 3, bottom panel). This fragment represents a polypeptide stretch beyond the
The fate of C-terminal portions of the Odz polypeptide were more difficult to document, due to a larger number of cleavages observed when antibodies directed against this region were used. Antibody 1329Y2 did not yield clear Western blots even after extensive purification steps on immunoaffinity and antigen-linked columns. In both cases, indications were that the C-terminal portion of the protein, truncated transgene had the capacity to encode an extensive degree, and that it is labile in the cell. In some cases, indications were that the C-terminal portion of the protein was cleaved to an extensive degree, and that it is labile in the cell.

**Cleaved Odz polypeptide fragments co-associate as heterodimeric subunits through cysteine bridges**

A number of lines of evidence, including data from fragments immunoprecipitated with one Odz antibody, then analysed by Western blotting with different Odz antibodies, suggested that the covalent association is with other extracellular fragments when probed with the same Odz antibodies. Cleaved Odz of 70 kDa was recognized both by mAb20 and 299EGF antibodies (and, as a 40 kDa further cleaved fragment, by mAb20) in mouse tissue extracts at reproducible sites, in the linear series shown in Figure 3 (bottom panel).

The overall conclusion, however, that Odz is cleaved multiple times at reproducible sites, in the linear series shown in Figure 3 (bottom panel).

**Two independent methods verify that Odz is a transmembrane protein with extracellular EGF repeats and a long intracellular C-terminal region**

Preliminary experiments showed that Odz is phosphorylated on tyrosine, supporting a type I transmembrane protein model of Odz, with a single transmembrane region between extra- and intracellular domains. Western-blot analyses were carried out on fly and cell-culture protein extracts prepared in the presence or absence of reducing agents (dithiothreitol or mercaptoethanol). A significant percentage, and in some cases nearly all, of the prominent low-molecular-mass Odz fragment bands disappeared under non-reducing conditions (Figure 6). The Odz-reactive material instead appeared as more-slowly migrating bands under non-reducing conditions when probed with the same Odz antibodies. Cleaved Odz of 70 kDa was recognized both by mAb20 and 299EGF antibodies (and, as a 40 kDa further cleaved fragment, by mAb20) in mouse tissue extracts at reproducible sites, in the linear series shown in Figure 3 (bottom panel).

The first approach was to biotin-decorate all extracellular protein domains on viable, non-permeabilized cells using sulpho-NHS-biotin [28, 29]. After neutralization of this reactive substrate and cell lysis under non-reducing conditions, biotinylated proteins were purified by avidin-based affinity chromatography, then examined by Western-blot analyses using the panel of Odz antibodies. As an example under reducing conditions, the 70/40 kDa fragments recognized by mAb20 (and the same

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**Figure 5** Mapping Odz protein fragments and mAb20 determinants using a C-terminal Odz truncation transgene

cDNA-based transgenes expressing a full-length Odz construct (FC) and a partial Odz construct of the 1120 N-terminal amino acids (PC) were placed under heat-shock promoter control. They were driven to expression by a 2 h heat shock (h.s.) in newly eclosed adults. Protein extracts of flies that underwent heat shock (+) or not (−) were subjected to PAGE followed by Western-blot analysis employing mAb20 anti-Odz monoclonal antibody. Extracts of yellow white flies without a transgene were run in parallel (YW), as were Schneider S2 cell protein extracts (S2).
Figure 6  Odz protein fragments associate under non-reducing conditions

Protein extracts were prepared and subjected to PAGE, then Western-blot analysis. Samples were prepared under reducing (red.) or non-reducing (non-red.) conditions (see the Experimental section). Black arrows point to Odz species associated entirely with larger complexes under non-reducing conditions, while arrows point to Odz fragment species which had a significant population with unchanged mobility under non-reducing conditions. (A) Association in pupae and Schneider S2 cells. Protein extracts were prepared from pupae and Schneider Cells and subjected to PAGE and Western-blot analysis with mAb20 anti-Odz antibody. (B) Association in Schneider cells; protein extracts were prepared and serial Western blots performed on the same filter, first reacted against polyclonal antibody 299EGF (left-hand panel), then stripped and reacted against mAb20 (right-hand panel). (C) Association in Schneider cells; protein extracts were prepared and serial Western blots were performed as in (B) but with 166ATM (left-hand panel) and then mAb20 (right-hand panel).

Figure 7  Mapping Odz protein fragments with respect to the cell membrane using reagents that modify extracellular polypeptides

(A) Accessibility of Odz fragments to extracellular reactive biotin. Intact Schneider cells were exposed to sulfo-NHS-biotin under conditions which allow only extracellular proteins to react with the reagent (see the Experimental section). After inactivation of the excess reagent, cells were harvested and crude protein extracts prepared. Avidin columns were used to purify all proteins covalently linked to biotin, then the purified fractions were subjected to PAGE and Western-blot analysis with antibodies mAb20 (left-hand panel), 489Y2 (middle panel) or 299EGF (right-hand panel). Crude Schneider protein extracts (crude S) before avidin-column purification were run on the same gels as the purification fraction (ipp biotin + ). A crude protein extract of embryos was run for comparison (embryo), as was a purified fraction of a Schneider crude extract from cells that had not been treated with sulfo-NHS-biotin (ipp biotin − ), to assess non-specific column binding.

(B) Accessibility of Odz fragments to extracellular proteases. Intact Schneider cells were exposed to extracellular proteases under conditions in which the cell membrane was not compromised (see the Experimental section). After protease inactivation, cells were harvested and crude protein extracts prepared, followed by PAGE and Western-blot analysis with antibodies mAb20 (left-hand panel), 489Y2 (middle panel) or 700EC (right-hand panel). Increasing time of exposure to protease is represented by the triangles above each panel, with exposure times of 0, 15, 30, 60 and 90 min to trypsin, or 60 min to proteinase K (60pk). Cell viability was validated by Trypan Blue exclusion.
70 kDa by 299EGF antibody) were biotinylated and were captured by the avidin column, indicating that they are extracellular (Figure 7A). The 45 kDa fragment recognized by mAb20 is also at least partially extracellular (Figure 7A). In contrast, the 75 kDa (and 65 kDa further cleaved) fragment recognized by the 489Y2 antibody (as well as by 166ATM) were not biotinylated, were not bound by the avidin column and therefore did not appear among the biotinylated proteins when probed with the 489Y2 antibody (Figure 7A). The 75 (and 65) kDa fragment is either intracellular, or less likely, buried in the membrane to the point of being inaccessible to biotin. In the same analysis, the less-abundant, full-length Odz was recognized by the 489Y2 antibody (Figure 7A), as would be predicted for all anti-Odz antibodies by all models, given that some portion of full-length Odz is present in the extracellular space. All of the Odz antibodies against protein regions N-terminal of the 166ATM region either recognized only extracellular (accessible to biotin) short fragments (299EGF and 700EC), or both extracellular and membrane-spanning fragments (accessible to biotin; 166ATM and mAb20). This places a transmembrane domain firmly between the 299EGF and 166ATM determinants (see Figure 7A).

An excellent correlation with these data was seen when a second method was employed to assess antigen-determinant position with respect to the cell membrane. In this independent method, live cells were treated with levels of protease in their medium titrated to achieve maximal levels of activity without compromising the cell membrane, assuring degradation of only extracellular proteins, or extracellular portions of transmembrane proteins [30]. The proteases were then neutralized, cells were lysed and Odz antibody Western-blot analyses were carried out to assess which detectable Odz polypeptides were susceptible to proteolysis, hence establishing their intracellular, extracellular or partially extracellular positions. As can be seen with increasing levels of trypsin and proteinase K, the 70/40 kDa fragment is fully susceptible to proteolysis, indicating that it is extracellular (Figure 7B). Contrast this with the 75 kDa fragment that is entirely resistant to the proteases in the medium exposed to the extracellular space (Figure 7B). These results are fully consistent with the results obtained by the biotinylation method (Figure 7B and results not shown).

In contrast, the 45 kDa fragment was largely unaffected by the proteases, with some diminution, and a slight downwards shift in mobility. This is in fact seen in the same lanes in which the 70/40 kDa fragments completely disappeared over the protease time course (Figure 7B, left-hand panel). As a result, we model this fragment as being transmembranal, with only its N-terminal portion extending into the extracellular space (Figure 8). The slight downward shift in mobility of the 45 kDa fragment represents less than a 5 kDa change, and appears as a smear, as might be expected for proteolysis of a short protein terminus accessible to protease action. We therefore model the 45 kDa fragment as ‘protruding’ only slightly into the extracellular space. At the same time, this fragment was very efficiently captured by avidin-column purification, despite only a limited extracellular surface that could be decorated by biotin moieties. However, the extracts purified on avidin columns were maintained under non-reducing conditions, and the efficiency of the 45 kDa fragment capture was significantly due to its cysteine-bridge attachment to extracellular Odz fragments (primarily 70 kDa; see Figure 8). The deployment of other portions of the protein, and the consistency of this data with that of other studies and with sequence motif landmarks, can be seen in the Discussion.

**DISCUSSION**

**A model of the Drosophila Odz protein as a type I transmembrane receptor**

By assessing molecular masses, position with respect to the cell membrane, association by cysteine linkages and other post-
translational modifications of processed Odz fragments, we have derived a model of Odz protein (Figure 8). The smallest resulting ‘end-point’ processed fragments remain the primary focus of the description, for brevity and simplicity. Yet, these end-point cleavage fragment sizes can be treated additively to combine to yield the larger fragment sizes expected from processing at less than the full complement of cleavage sites. The sizes are consistent and correlated between the results of all of the antibodies used.

The most N-terminal fragment described, of 70 kDa (a subspecies of 40 kDa), is a fully extracellular Odz fragment accessible to the extracellular protein-modifying agents, reactive biotin and proteases (Figure 8). We have also determined that this is the only fragment characterized that is glycosylated, with mannose-containing moieties, as demonstrated with concanavalin A–horseradish peroxidase conjugate binding (results not shown). This is consistent with its predicted glycosaminoglycan attachment consensus sites and extracellular assignment. The fragments’ N-terminus is mapped to the most N-terminally located cleavage site in the model (Figure 8, cleavage a). This falls at a position in the sequence corresponding to the highly hydrophobic N-terminal stretch predicted to be a signal peptide [3,4]. In fact, all Odz fragment sizes recognized by the N-terminally directed antibodies are consistent with a cleavage at this prospective signal-peptide site (Figure 3, bottom panel, cleavage a). The observed size of the 70 kDa fragment is consistent with a protein fragment spanning from the signal peptide to a point shortly before the second hydrophobic, membrane-spanning stretch (see Figure 8), predicted by the sequence. The 70 kDa fragment is modelled as a discrete portion of the protein positioned to contact Odz-interacting proteins via the EGF-like repeats.

Located immediately C-terminally is a 45 kDa fragment of Odz spanning the plasma membrane (Figure 8). This is a processed product with only a small terminal portion accessible to extracellular protease. Its efficient capture on avidin columns after Odz reaction with extracellular reactive biotin was at least partially dependent on mediation by a cysteine-linked 70 kDa fragment under non-reducing conditions. The placement of the bulk of the 45 kDa fragment in the intracellular space is also fully consistent with the additional post-translational modifications detected. It is not glycosylated with mannose-containing moieties, and is phosphorylated on tyrosine (results not shown, and [3]). Its association with the extracellular 70 kDa processing product is supported by the paired antibody assessments performed. Whereas the 45 kDa species is the end-point product when all cleavage sites are utilized, this fragment is modelled as reaching the cell surface as part of a longer processed product, including the continuation of the C-terminus of Odz.

The sequence of the 45 kDa protein fragment overlaps with a further C-terminal end-point cleavage Odz fragment of 65 (or 75) kDa. This processing product is completely inaccessible to the extracellular protein modifiers tested. The 65/75 kDa fragments are tyrosine-phosphorylated, like the 45 kDa fragment, and are not glycosylated, like the 70 kDa (40 kDa) fragment (results not shown). As a result of the large number of species detected, we did not model the protein beyond the 489Y2 determinants, or the N-terminal half of the protein (see Figure 8). Instead, the C-terminal half of the Odz protein, which all antibodies show as being reduced to very small fragments, is seen by us to be altered by a very large number of cleavages or, possibly, degradation within the cell (1329Y3/4 and 912Y5; results not shown). The overview is a type I protein that is delivered to the plasma membrane after extracellular cleavage. By this model, the heterodimeric protein of two subunits linked by a cysteine bridge is further cleaved intracellularly, perhaps in concert with its signalling function.

**N-terminal membrane-anchor findings and other Odz/Ten-m family protein models**

The strongest hydrophobic stretch within all Odz/Ten-m proteins occurs 250–400 amino acids from the predicted start codons of every known homologue. These stretches are interpreted as signal peptides (Figure 8) [3,4,14,17], or as transmembrane domains [9,11,15]. The presence of large polypeptide domains (30–50 kDa) before this hydrophobic stretch mark these domains as unusually long to be slatted for degradation as ‘pre-signal’ domain sequences. Furthermore, as homologues in additional species are discovered, it has become clear that sequences are weakly conserved between the different homologues. This makes their fate as degraded leaders even more unlikely. In this study, however, we did not prepare any antibodies or analyse any data pertaining to this region.

Previous work on mouse homologue Ten-m2 (Odz2) provided evidence that the very N-terminal portion of the protein is not discarded, is anchored by the N-terminal hydrophobic stretch and is intracellular [11]. This evidence underpins the argument that Ten-m2 is therefore a type II transmembrane protein, and the authors argue that the C-terminal 90% of the protein is extracellular. The second, weaker, transmembrane stretch we predict in Odz/Ten-m proteins is considered in that analysis to be insufficient to act as a membrane-spanning domain. In contrast, our model does not directly treat the fate and position of this N-terminal end of the protein. Rather, it is mapped to be cleaved from the rest of the Odz polypeptide, which is then inserted into the membrane as a type I protein via the second transmembrane stretch. Our model and that of Ten-m2 [11] do concur in placing the EGF-like repeats extracellularly. However, besides the role of cleavage, the core difference in the models is based on our strong evidence that the C-terminal 70% of the Odz/Ten-m proteins (including tyrosine-phosphorylated protein fragments) are intracellular, showing that the second hydrophobic stretch does span the membrane. In fact, in the case of *Drosophila* Odz, our reactive biotin and protease-digestion approaches indicated that there is no prominent cell-associated C-terminal domain species of Odz that are extracellular. While it is unprecedented, it is possible that both Odz hydrophobic stretches act a membrane-spanning anchors. After cleavage, Odz/Ten-m proteins could be presented on the plasma membrane with an N-terminal portion oriented as a type II polypeptide, and the C-terminal portion as a type I polypeptide, with or without co-association.

It is also possible to reconcile these mutually exclusive descriptions by proposing that *Drosophila* Odz/Ten-m and Ten-m2 (Odz2) of mammals are deployed differently with respect to the cell membrane. This is not likely, given the highly conserved nature of the Ten-m/Odz gene family. Alternatively, the fact that the homologues, especially those of the mammalian species, have splice variants, makes it possible that alternative protein model forms exist in animals of both phyla, but that the type I form predominates in *Drosophila* while the type II protein predominates in mice. In any case, an Odz protein deployed to two different sites is assumed to signal to two very different manners, with the EGF-like domain contacting partner proteins in the extracellular space perhaps being the only mode of action common between the models. Additional reagents and approaches must be applied to resolve discrepancies between these models, especially for the extreme N-terminal and C-terminal regions of the proteins.
Possible functional implications of type I Odz structure

The sequences encoding the N-terminal stretch, hydrophobic domains, EGF repeats and tyrosine-phosphorylation consensus protein sequences are present in all Odz/Ten-m family proteins in the same positions, and are therefore likely to function in the same manner in all species. In genome-wide comparisons, the odz/Ten-m gene family shares no significant homology with any other eukaryotic genes outside of the EGF repeats. Therefore, based on its protein sequence alone, it is a truly unique protein class, sharing only the closest EGF homology with a number of very different transmembrane (Notch, Serrate and Crumbs) and extracellular-matrix (tenascin) protein families. Nonetheless, based on its protein structure as a heterodimeric type I protein deployed as outlined in this paper, there are structural similarities to the protein Notch (among the families mentioned above) suggested, which might imply functional conservation and mechanism similarities. Both Notch and Odz/Ten-m are cleaved before arriving at the cell surface, and are then deployed with the EGF-repeat bearing regions anchored to the cell via S-S cysteine bridges to more C-terminal domains as heterodimeric type I transmembrane proteins [26,31]. Just as in for the case for Notch, uncleaved forms of Odz which are inaccessible to extracellular modifying reagents, which represent forms of the protein in transit in the endoplasmic reticulum and Golgi en route to the cell surface, are detectable [26]. Most importantly, there are subsequent C-terminal cleavages of Notch which potentiate its primary mode of signalling [32], with tyrosine phosphorylation as only an additional secondary modulation. While no evidence exists that any portion of Odz translocates to the nucleus, at least the intracellular cleavages are similar to those of Notch, and might share mechanistic conservation. This will be an important starting point for asking further questions about the function of Odz. As one indication of the potential of these modifications to embody events mediating signalling, we see that Odz at different developmental stages, pupal and embryonic, is cleaved and phosphorylated to different extents. These stage-sensitive post-translational events might represent modifications involved in signalling by the protein, which change with signalling status.

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REFERENCES

4 Baumgartner, S., Martín, D., Hagios, C. and Chiquet-Ehrismann, R. (1994) ten-m, a Drosophila gene related to tenascin, is a new pair-rule gene. EMBO J. 13, 3728–3740

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