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The evolutionarily conserved *porcupine* gene family is involved in the processing of the Wnt family

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The *Drosophila* segment polarity gene product Porcupine (Porc) was first identified as being necessary for processing Wingless (Wg), a *Drosophila* Wnt (Wnt) family member. Mouse and *Xenopus* homologs of *porc* (*Mporc* and *Xporc*) were identified and found to encode endoplasmic reticulum (ER) proteins with multiple transmembrane domains. In contrast with *porc,* four different types of *Mporc* and *Xporc* mRNA (A–D) are generated from a single gene by alternative splicing. *Mporc* mRNA is differentially expressed during embryogenesis and in various adult tissues, demonstrating that the alternative splicing is regulated to synthesize the specific types of Mporc. In transfected mammalian cells, all Mporc types affect the processing of mouse Wnt 1, 3A, 4, 6, and 7B but not 5A. Furthermore, all Mporc types are co-immunoprecipitated with various Wnt proteins. These results suggest that Mporc may function as a chaperone-like molecule for Wnt. Interestingly, all Mporc types can substitute for Porc, as they are able to rescue the phenotypes of *Drosophila porc* embryos. Consistent with this observation, Mporc, like Porc, modifies the processing of Wg expressed in mammalian cells. These results demonstrate that the *porc* gene family encodes the multitransmembrane ER proteins, which are evolutionarily well conserved and involved in processing the Wnt family.

Keywords: alternative splicing; chaperone; Porcupine; Wnt family.

The development of multicellular organisms (embryogenesis and organogenesis) is a complex process, which requires formation of different types of cell in a precise order. Cell–cell interactions through secreted short-range and long-range signaling polypeptides and their receptors have been shown to have important roles in such events. A number of signaling polypeptides have been identified and classified into several families (Wnt, Hedgehog, FGF, TGF-α, and TGF-β) on the basis of their structures.

The *Wnt* family encodes secreted glycoproteins of molecular mass ≈ 40 kDa. A number of *Wnt* genes have been identified in vertebrates, *Drosophila*, and *Caenorhabditis elegans*, and shown to have critical roles in the decision about cell fate and behavior at different stages of development. The *Drosophila* ortholog of *Wnt-1* is the segment polarity gene *wingless* (*wg*) which is the best-characterized member of the family [1].

The secretion and processing of Wnt proteins has been studied with tissue cultured cells transfected with various Wnt cDNAs [2,3]. Processing of Wnt is inefficient in most cell types, as multiple processing intermediates are present. As a result, Wnt is not secreted efficiently outside of cells and most of the protein is retained in the endoplasmic reticulum (ER) associated with an HSP70 protein, BiP [4]. *wg* mutants with lesions in the secretion and transport components have been identified [5–8], suggesting that Wg processing and secretion is also complex in *Drosophila*. The biochemical [9] and genetic [10–14] evidence suggests that secreted Wg interacts with heparan sulfate proteoglycans in the extracellular matrix or at the cell surface. This interaction is critical for efficient signaling and may also affect diffusion of Wg away from synthesizing cells. These results indicate that the processing, secretion, and transport of Wnt are complex involving a number of specific factors.

One of the *Drosophila* segment polarity genes, *porcupine* (*porc*), encodes a multipass transmembrane ER protein, which is required for normal distribution of Wg in embryos. Porc stimulates the processing of Wg when expressed in *Drosophila* cells *in vitro* [15] and is also necessary for the localization of *Drosophila* Wnt-3 [16] on the axon tracts of the embryonic central nervous system (K. Tanaka and T. Kadowaki, unpublished results). As Wg signaling components are well conserved among multicellular organisms, Porc homologs must be present in other species. In fact, the *C. elegans porc* homolog, *mom-1*, was identified in a search for maternal genes necessary for endoderm formation [17,18]. Mom-1 is necessary in Mom-2 (Wnt)-producing cells in the same way as Porc is required in Wg-synthesizing cells.

In this study, we identified vertebrate (mouse and *Xenopus*) homologs of *porc* and characterized their structures, functions, and patterns of expression during fetal and adult stages of development. The results demonstrate that the *porc* gene family encodes the evolutionarily conserved ER membrane proteins involved in processing the Wnt family.

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Abbreviations: ER, endoplasmic reticulum; dpc, days post coitum; Porc, *Drosophila* segment polarity gene product Porcupine; Wg, Wingless.

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MATERIALS AND METHODS

Isolation of *Xporc* and *Mporc* cDNAs

A partial 563-bp MG61 (GenBank accession no. L08239) cDNA was isolated by PCR with a human retina cDNA library

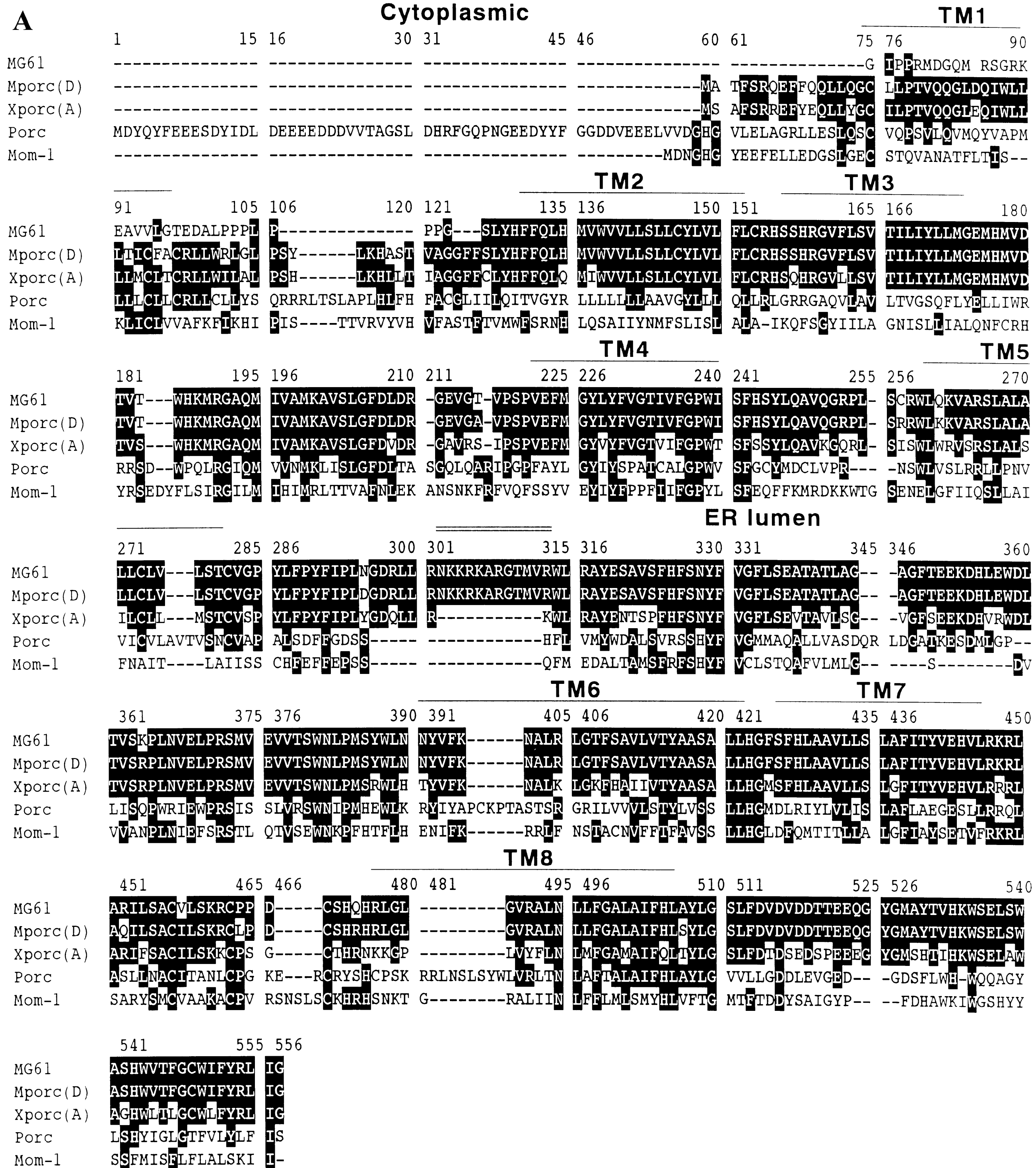


Fig. 1. Amino-acid sequences and Kyte–Doolitle hydrophobicity plots of Porc family members. (A) The amino-acid sequences of human MG61, Mporc (D), Xporc (A), *Drosophila* Porc, and *C. elegans* Mom-1 are aligned. Identical amino acids are indicated by a solid box background. Similarity is found throughout the entire sequence of the members including the transmembrane domains. Porc has an extra hydrophilic N-terminal sequence. The N-termini of the members are directed towards the cytoplasm and the positions of the eight transmembrane domains (TM1–8) are indicated by single lines above the amino-acid sequence. In contrast with other members, MG61 and Mporc (D) have a stretch of amino acids (NKKRKARGTMV, shown by a double line) in the ER luminal domain. (B) Analysis of Porc family protein structures by a Kyte–Doolitle hydrophobicity plot. All have similarly positioned transmembrane domains and appear to lack the signal sequences for ER targeting. The region affected by alternative splicing is the hydrophilic domain in the middle of Mporc (shown by an arrow) and Xporc. This is a part of the largest domain facing the ER lumen (indicated by a bar).

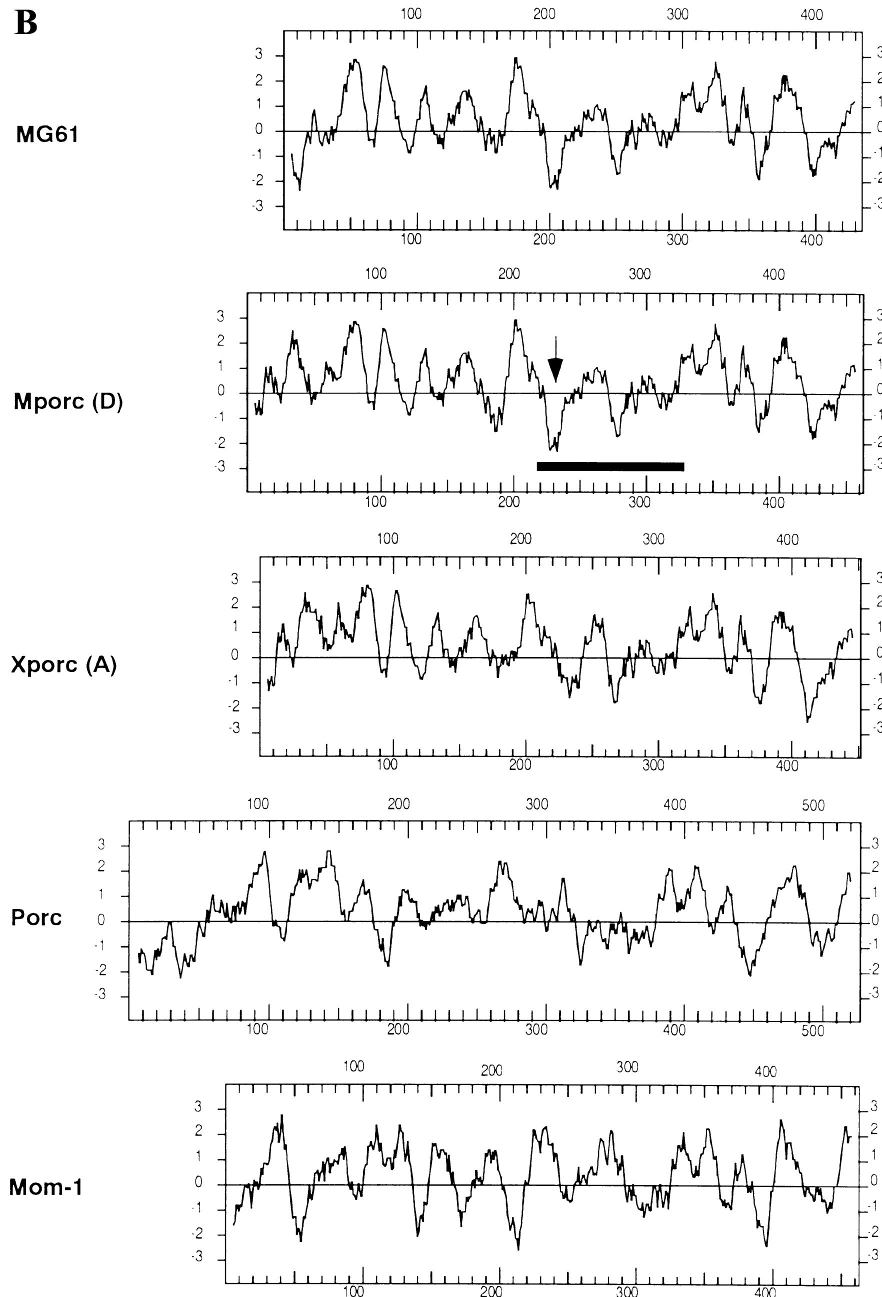


Fig. 1. continued.

as a template and the following primers: 5′-CGGAATTCGG-CACCATGGTAAGGTGGCTGCGAGCCTAC-3′ and 5′-CGG-GATCCGTCATCCACATCGACATCAAACAGGGA-3′. The PCR product was cloned in pBluescript (pBS) II and used as a probe to isolate 1.6-kb MG61cDNA from the same library. The 1.6-kb MG61cDNA was used to screen *Xenopus laevis* oocyte and mouse embryo cDNA libraries under low-stringency conditions with 6 × NaCl/P_i/EDTA containing 25% formamide at 42 °C. The membranes were washed with 2 × NaCl/Cit/0.1% SDS at room temperature followed by 6 × NaCl/Cit/0.1% SDS at 50 °C. Four overlapping clones and one clone related to MG61were isolated from *Xenopus* oocyte and mouse embryo cDNA libraries, respectively. A 0.9-kb *Mporc* cDNA was used as a probe to screen a mouse brain cDNA library to isolate its full-length cDNA.

An *Mporc* genomic DNA clone was isolated from a 129/SV genomic DNA library with the 5′ portion of *Mporc* cDNA as a probe. The DNA fragments containing *Mporc* cDNA sequence were cloned in pBSII and partially sequenced.

RT-PCR and RNase-protection assay

For analysis of the pattern of expression of Mporc by RT-PCR, cDNA was first synthesized with 2 μg total RNA from mouse embryos and various tissues, oligo(dT) primer, and reverse transcriptase. PCR was carried out for 30 cycles with cDNA synthesized as above and the following set of primers: 5′-GCCTTGTACTGTCCACTTGTGTGGG-3′ and 5′-AGGAGACAGCACTCTCGTAGGCTCG-3′. To construct full-length *Mporc* (*A–C*) cDNAs, RT-PCR was performed

as above except that the following set of primers was used: 5′-TACCTCTACTTTGTGGGCACCATCGTCTTT-3′ and 5′-ATGGACCGGGGCAGCTCCACATTCAACGGT-3′. The RT-PCR products were digested with *Eco*RV and *Xba*I and the resulting DNA fragments were cloned in *Mporc* (*D*) cDNA at the same restriction enzyme sites. *Xporc*-spliced variants were identified by RT-PCR with total brain RNA and the following primers: 5′-TACCTCTTCCCCTACTTCATCCCAC-3′ and 5′-ACAAAGTAATTGCTGAAGTGGAACG-3′. The RT-PCR products were cloned in pBSII and sequenced. For the RNase-protection assay, a 502-nt riboprobe was synthesized with plasmid carrying *Mporc* *Eco*RV–*Bgl*II fragment and then gel purified. A 10 μg portion of total RNA and the riboprobe corresponding to 2×10^4 c.p.m. were dissolved in hybridization buffer and incubated at 45 °C for 12 h. The mixture of RNase A and T1 was added to the reaction, and the protected fragments were then analyzed by denaturing PAGE.

RNA injection

Capped *porc* and *Mporc* (*A–D*) RNA was prepared by *in vitro* transcription. The size and quantity of each capped RNA was analyzed by gel electrophoresis. The same amount of RNA was injected into precellular blastoderms. The embryos were derived from females with homozygous $svb^{YP17b}porc^{PB16}$ germline clones crossed with wild-type males. *porc* mutant embryos rescued as a result of RNA injection showed *svb* phenotype. The injected embryos were allowed to develop at 18 °C and their cuticles examined after 3 days. In these experiments, germline clones were generated using the flipase-dominant female sterile (FLP-DFS) technique [19].

Western blotting

Human 293 cells were transfected with *wg* alone and *wg* in addition to either *porc* or *Mporc* (*A–D*). The cell lysates were prepared 2 days after transfection and separated by SDS/PAGE (10% gel) and then blotted to nitrocellulose membrane. The membrane was incubated with 10 000-fold-diluted rabbit anti-Wg IgG and then with 3000-fold-diluted horesradish peroxidase-conjugated donkey anti-(rabbit IgG). The signal was detected by the ECL system (Amersham).

Intracellular localization of Mporc

To construct HA-epitope-tagged *Mporc* (*A–D*), a *Not*I site was first introduced after the first ATG codon, and then the *Not*I DNA fragment carrying three HA epitopes [20] was cloned. These HA-epitope-tagged cDNAs were cloned in a mammalian expression vector, pMKIT [21]. COS-1 cells were transfected and, 2 days after transfection, the cells were fixed, permeabilized, blocked, and incubated with 12CA5 mouse monoclonal antibody specific for HA epitope (80-fold dilution) and rabbit anti-calnexin IgG [22] (50-fold dilution). Tetramethylrhodamineisothiocyanate-conjugated anti-(mouse IgG) (rabbit serum adsorbed) and fluorescein isothiocyanate-conjugated anti-rabbit IgG (mouse serum adsorbed) were used for signal detection with 200-fold dilutions.

Processing of mouse Wnt proteins in the presence of Mporc (A–D)

Mouse *Wnt 3A*, *4*, and *7B* were tagged with single c-*myc* epitopes at their C-termini. Mouse *Wnt 1*, *5A*, and *6* were tagged with triple c-*myc* epitopes (three c-*myc* epitopes in tandem) at the amino-acid positions 57, 153 and 335, respectively. The c-*myc*-epitope-tagged *Wnt* and *Mporc* (*A–D*) cDNAs were cloned in the pMKIT. COS-1 cells were transfected with each *Wnt* in the absence and presence of either *Mporc* (*A–D*). Two days after transfection, the cells were labeled with [^{35}S]methionine (0.2 mCi·mL^{-1}) for 30 min and then solubilized with RIPA buffer. Immunoprecipitation was carried out with rabbit polyclonal rabbit anti-Myc IgG and Protein A–Sepharose, and the immunoprecipitates were washed five times with RIPA buffer. The labeled Wnt proteins were separated by SDS/PAGE (10% gel) and then analyzed with a laser image analyzer BAS2000 (Fuji). The radioactivity of individual bands was quantified and the ratio of the largest band to the sum of the others in each lane was calculated for different Wnt proteins. These experiments were repeated at least twice and a similar result was obtained each time.

To investigate binding of Mporc (A) to various Wnt proteins, COS-1 cells were transfected with expression constructs for HA-tagged Mporc (A) and either Myc-tagged Wnt or partial laminin β chain [23]. Two days after transfection, the cells were solubilized with RIPA buffer and then 0.2 vol. of cell lysate was adjusted to 1 × SDS/PAGE sample buffer to use as total cell lysate. The rest of the cell lysates were immunoprecipitated with anti-Myc IgG and the immunoprecipitates then Western blotted with 12CA5 mouse monoclonal antibody (specific for HA epitope) along with total cell lysates.

RESULTS

Isolation of vertebrate homologs of *porc*

A database search revealed that human MG61 sequence has significant similarity to *porc* [15]. The 1.6-kb MG61 cDNA was first isolated from a human retina cDNA library. With this cDNA as a probe, *Xenopus laevis* oocyte and mouse embryo cDNA libraries were screened by low-stringency hybridization. A 1.6-kb cDNA with an ORF capable of encoding a 51.6-kDa protein was isolated from the *Xenopus* oocyte cDNA library and is referred to as *Xporc* (*A*). A partial 0.9-kb cDNA was first isolated from mouse embryo cDNA library and then used to screen a brain cDNA library. A 1.9-kb cDNA with an ORF capable of encoding a 52.5-kDa protein was isolated and is referred to as *Mporc* (*D*). These cDNAs appear to be full length because each corresponds to the size of mRNA detected by Northern blot (not shown). No other *porc*-related cDNAs with significant similarity were obtained by this procedure. The amino-acid sequences of human MG61, Mporc (D), Xporc (A), Porc, and *C. elegans* Mom-1 were aligned as shown in Fig. 1A. The three vertebrate Porc homologs are well conserved, with extensive similarity to each other (Fig. 2). *Drosophila* Porc has an extra hydrophilic N-terminal sequence, which is not found in

IDENTITY \ SIMILARITY	MG61	Mporc(D)	Xporc(A)	Porc	Mom-1
MG61		99	87	67	59
Mporc(D)	97		93	67	58
Xporc(A)	74	75		66	61
Porc	47	48	45		69
Mom-1	44	42	42	39	

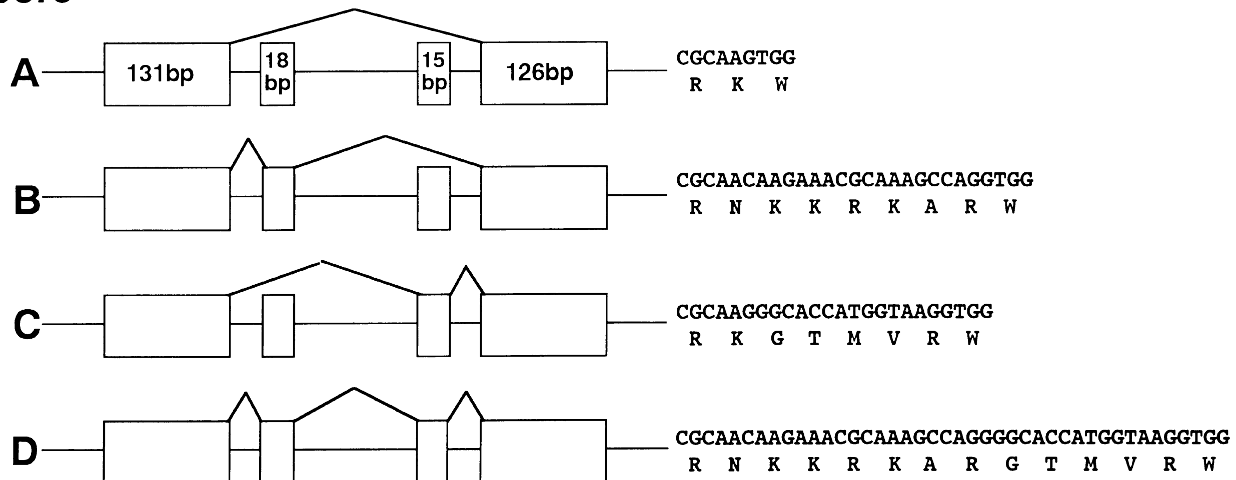
Fig. 2. Identity and similarity of Porc family members. The amino-acid sequences of MG61, Mporc (D), Xporc (A), Porc, and Mom-1 are compared. Percentage identity is indicated to the left of the diagonal line and similarity to the right.

the others (Fig. 1A). All members appear to lack the signal sequences for ER targeting and have the same topology in the ER membrane (with N-terminus facing the cytoplasmic side) based on the charge difference surrounding the transmembrane domains. A Kyte–Doolitle hydrophobicity plot [24] of each protein is shown in Fig. 1B. These data suggest that members of the *Porc* family encode membrane proteins with multiple (eight) transmembrane domains at conserved positions. No other specific functional domains were found by a database search.

Alternatively spliced variants of *Mporc* and *Xporc* mRNA

The sequence alignment shown in Fig. 1A reveals that human MG61 and Mporc (D) have an extra 11 amino acids (NKKRKARGTMV) at 302 −312 (indicated by double lines in Fig. 1A), which are absent from the others. We therefore asked whether *Mporc* cDNA not encoding this stretch of amino acids is also present. *Mporc* cDNA from different tissues was analyzed by RT-PCR with two primers flanking this region (see Materials and methods). As shown in Fig. 4B, three discrete bands were detected. By sequencing these PCR products, the largest band was found to correspond to *Mporc* (*D*), the medium band to *Mporc* (*B*) and (*C*), and the smallest to *Mporc* (*A*). Between arginine (at position 228) and tryptophan, *Mporc* (*A*) encodes K, which is identical with *Xporc* (*A*). *Mporc* (*B*) and (*C*) encode NKKRKAR and KGTMVR, respectively. To prove these four types of mRNA are generated by alternative splicing, a part of the *Mporc* genomic DNA was sequenced (Fig. 3A). This region of mRNA is encoded by two exons (18 and 15 bp) separated by ≈ 900 bp. The inclusion of both exons produces *Mporc* (*D*). The inclusion of either upstream 18 bp exon or downstream 15 bp exon results in *Mporc* (*B*) or (*C*). *Mporc* (*A*) is generated by skipping these two exons.

Similarly, three additional types of *Xporc* mRNA (*B–D*) were identified by RT-PCR. All types of cDNA and their predicted amino-acid sequences are shown in Fig. 3B. The amino-acid sequence of each type is well conserved between mouse and *Xenopus*, suggesting that the generation of four types of *porc* transcript by alternative splicing is conserved in vertebrates. In contrast, *Drosophila porc* and *C. elegans mom-1* mRNAs do not have any variations because there is no intron in this region of genomic DNA. On the basis of sequence



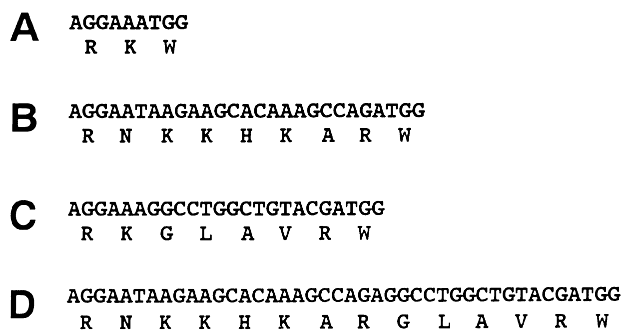


Fig. 3. Four types of *Mporc* and *Xporc* mRNA generated by alternative splicing. (A) Schematic representation of the relevant part of *Mporc* genomic DNA and *Mporc* (*A–D*) cDNA sequences and their predicted amino-acid sequences. Two exons (18 and 15 bp) are spliced with four different patterns to produce *Mporc* (*A–D*) mRNAs. The distance between the 131 and 18 bp exon is 115 bp, between the 18 and 15 bp exon is ≈ 900 bp, and betweeen the 15 and 126 bp exon is 88 bp. All Mporc types have arginine and tryptophan in common. (B) The relevant part of *Xporc* (*A–D*) cDNA sequences and their predicted amino-acid sequences. All Xporc types have arginine and tryptophan in common. The corresponding Mporc and Xporc types have similar amino-acid sequences.

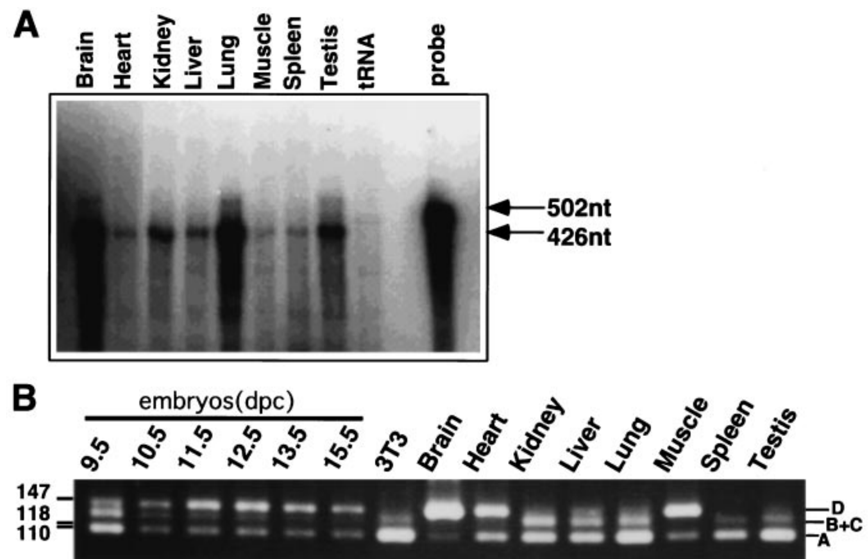


Fig. 4. Pattern of expression of *Mporc* at fetal and adult stages of development. (A) The expression of all *Mporc* types in various adult tissues was analyzed by the RNase-protection assay. *Mporc* is expressed in all tissues examined and most abundantly in brain and lung. The size of the riboprobe and protected fragment are 502 and 426 nt, respectively. (B) The expression of *Mporc* (*A–D*) mRNA during embryogenesis and in various adult tissues was analyzed by RT-PCR. During embryogenesis, the ratio of the expression levels of types *A, B* + *C*, and *D* changes over 9.5–11.5 dpc and remains constant later (up to 15.5 dpc) (see the text for details). The types of *Mporc* mRNA expressed in various tissues are different. Type *D* is abundant in brain, heart, and muscle. Type *A* is abundant in the rest of the tissues. Mouse 3T3 fibroblasts express multiple types of *Mporc* mRNA (type *A* and either *B* or *C* at least), suggesting that alternative splicing can occur in a single cell. The size of the molecular-mass marker is shown on the left in base pairs.

alignment (Fig. 1A), *porc and mom-1* cDNAs appear to correspond to vertebrate Porc (A).

Pattern of expression of *Mporc* during fetal and adult stages of development

Mporc expression in different adult tissues was analyzed by the RNase-protection assay, which detects all types of the transcript. *Mporc* mRNA was found to be expressed in all tissues examined, being particularly abundant in brain and lung (Fig. 4A), where many *Wnt* family members are expressed [25]. The types of *Mporc* mRNA expressed in various tissues were also analyzed by RT-PCR. As shown in Fig. 4B, type *A* is abundant in kidney, liver, lung, spleen, and testis. Type *D* is expressed least in these tissues, but is abundant in brain, heart, and muscle. These results demonstrate that the alternative splicing of *Mporc* is tissue specifically regulated to synthesize the particular types of Mporc. As the mouse 3T3 fibroblast cell line appears to express at least type *A* and either *B* or *C*, a single cell can express multiple types of *Mporc* mRNA. It is therefore unlikely that a single cell expresses one type of *Mporc* mRNA in various tissues.

The ratio of the different types of *Mporc* mRNA varies during embryogenesis (Fig. 4B). The ratio of type *A*, *B* + *C*, and D transcripts is 2, 1.5, and 1 respectively in mouse embryos 9.5 days post coitum (dpc), 2.5, 1, and 3 at 10.5 dpc, and 2.5, 1, and 5 at 11.5, 12.5, 13.5, and 15.5 dpc. The different types of *Mporc* mRNA are also produced by regulated alternative splicing during mouse embryogenesis.

The function of the *porc* gene family is conserved

Although the structures of Porc and its vertebrate homologs are quite similar, it does not necessarily indicate that they also have the same functions. *Drosophila* embryos that lack both maternal and zygotic *porc* function (referred to as *porc* embryos) show the same segment polarity phenotypes as *wg* embryos [15]. These embryos lack the naked posterior half of each segment, denticle diversity, segmental furrows, and terminal structures (compare Fig. 5A with B). Figure 5C shows the cuticle of a *shavenbaby* (*svb*) embryo, which has fewer denticles than the wild-type embryo. The remaining denticles are small and have dot-like morphology. They are arranged in 'belts', with one belt per abdominal segment [26] (compare Fig. 5A with C). These embryos have a normal segmentation pattern and the terminal structures, for example, Filzkörper materials (shown by arrows). The naked region is seen in *svb porc* double mutant embryos (Fig. 5D) because of the effects of the *svb* mutation described above. The *svb* marker is necessary to distinguish *svb porc* embryos rescued by RNA injection (with *svb* phenotype) from paternally rescued wild-type embryos (without *svb* phenotype). The naked cuticle, segment boudaries, and tail structures except denticle diversity (because of *svb* mutation) can be restored by *Drosophila porc* RNA injection in these embryos (Fig. 5E). The head structure was often not rescued, probably because RNA was injected into the posterior end of the embryos. This assay therefore allowed us to test whether *Mporc* can replace the functions of *porc* in *Drosophila* embryos. The capped RNA of *Mporc* (*A–D*) was prepared and injected into *svb porc* precellular blastoderm embryos. As shown in Fig. 5F–I, the posterior spiracles with Filzkörper materials (shown by arrows) were present in these RNA-injected *svb porc* embryos. In addition, some embryos developed the segmentation pattern of *svb* at the end of the abdominal segments (Fig. 5F). However, the extent of rescue was significantly less than that achieved by injection of *porc* RNA (for example, compare Fig. 5C,E, and F). These results nevertheless suggest that the function as well as the structure of *Porc* family members is evolutionarily conserved.

Drosophila Porc is localized at the ER when expressed in *Drosophila* cells as an epitope-tagged protein [15]. Each *Mporc* (*A–D*) was tagged with HA epitope at its N-terminus after the initiation methionine and expressed in COS-1 cells. Like Porc, all types of Mporc were concentrated at the ER. Figure 6A shows the localization of Mporc (D), which is identical with that of the ER-resident protein, calnexin.

To investigate the conservation of function of *Porc* family members *in vitro*, the effect of Mporc (A–D) on the processing of Wg was examined in human 293 cells by Western blot. As previously shown [15], form I represents the signal-sequence-

cleaved but not N-glycosylated Wg, which co-migrates with Wg synthesized in the presence of tunicamycin. Forms II, III, and IV appear to have one, two, and three *N*-glycan chains, respectively. These N-glycosylated forms of Wg are sensitive to endoglycosidase H. In addition, forms II–IV are synthesized when *wg* mRNA is translated *in vitro* with rabbit reticulocyte lysates in the presence of dog pancreas microsomes (data not shown). As this *in vitro* system only supports the addition of *N*-glycans in the ER, forms II–IV must have different numbers of *N*-glycan chains rather than differentially processed *N*-glycans through the secretory pathway. As shown in Fig. 6B, the abundance of forms II, III, and IV was equal in the cells transfected with *wg* alone, but the most glycosylated form, IV, icreased in the cells transfected with *wg* and either *porc* or *Mporc* (*A–D*). These results are consistent with above data demonstrating that *Mporc* is able to rescue the phenotypes of *Drosophila porc* embryos. The pattern of glycosylation of Wg appears to be the same in mammalian cells as in *Drosophila* S2

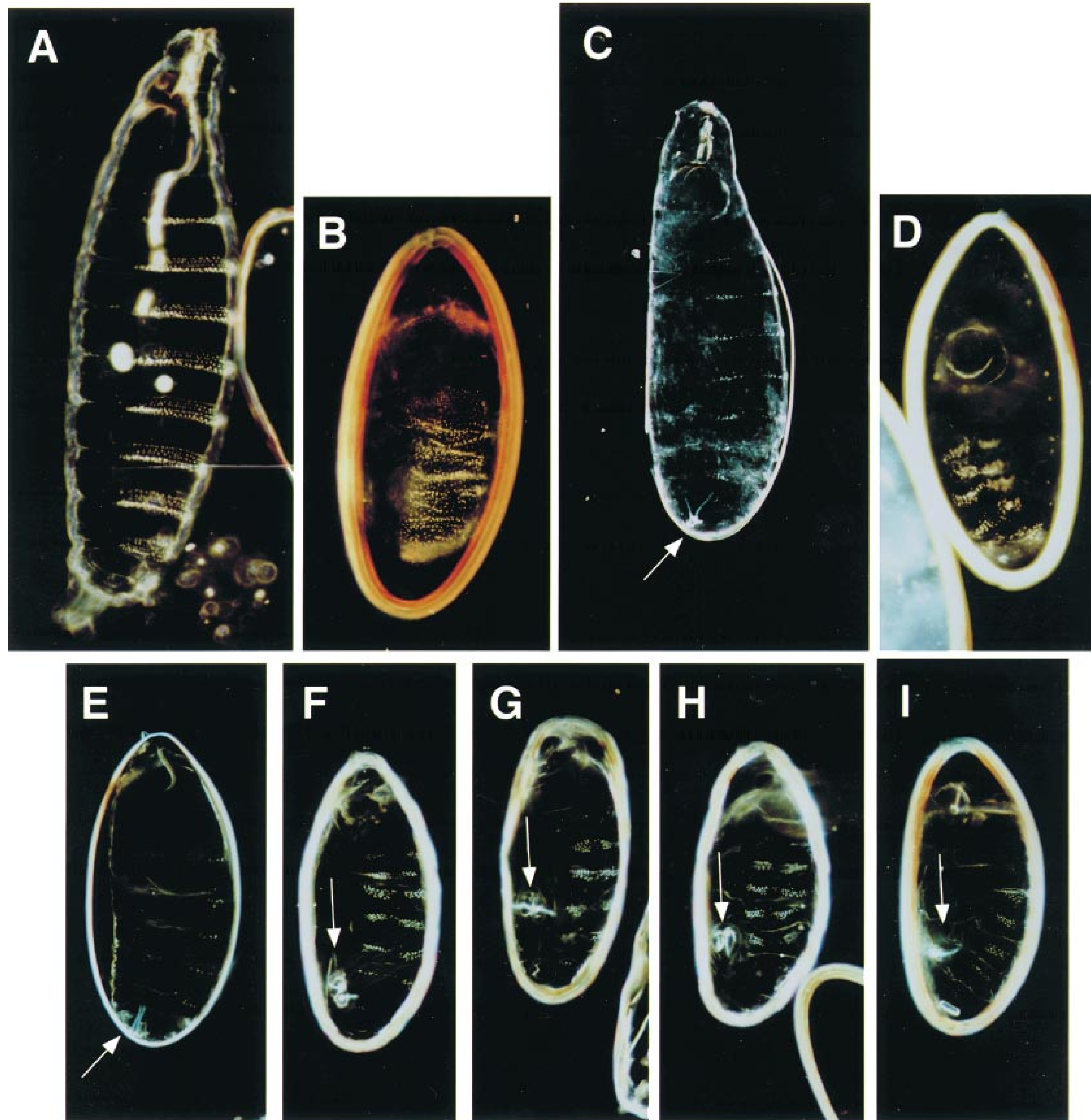


Fig. 5. Rescue of *Drosophila porc* embryos by *Mporc* RNA injection. The cuticles of wild-type (A), *porc* (B), *svb* (C), *svb porc* (D), *svb porc* embryos injected with *porc* (E), *Mporc* (*A*) (F), *Mporc* (*B*) (G), *Mporc* (*C*) (H), and *Mporc* (*D*) (I) RNA are shown. The wild-type embryos have a naked cuticle in each posterior segment, cells secreting different types of denticles in each anterior segment (resulting in denticle diversity), segmental furrows, and terminal (head and tail) structures (A). The *porc* germline clone embryos completely lack the above structures, and their abdominal segments are covered with denticle (B). The *svb* embryos develop normally except that they have denticles with reduced number and size (C). The *svb porc* double-mutant embryos have the naked region because of the effect of *svb* mutation (D). These embryos also lack a normal segmentation pattern and the terminal structures. The injection of *porc* RNA into *svb porc* embryos shifts the phenotypes to those of *svb* embryos (E). The head structure is often not rescued because RNA was injected to the posterior end of the embryos. The injection of *Mporc* (*A–D*) RNA into *svb porc* embryos partially rescued the *porc* phenotypes in the posterior part of the embryos (appearance of *svb* denticle phenotype and posterior spiracles including Filzköper materials; F–I). The extent of the rescue by *Mporc* RNA is, however, less than that by *porc* RNA (compare E with F–I). Filzköper materials are indicated by arrows.

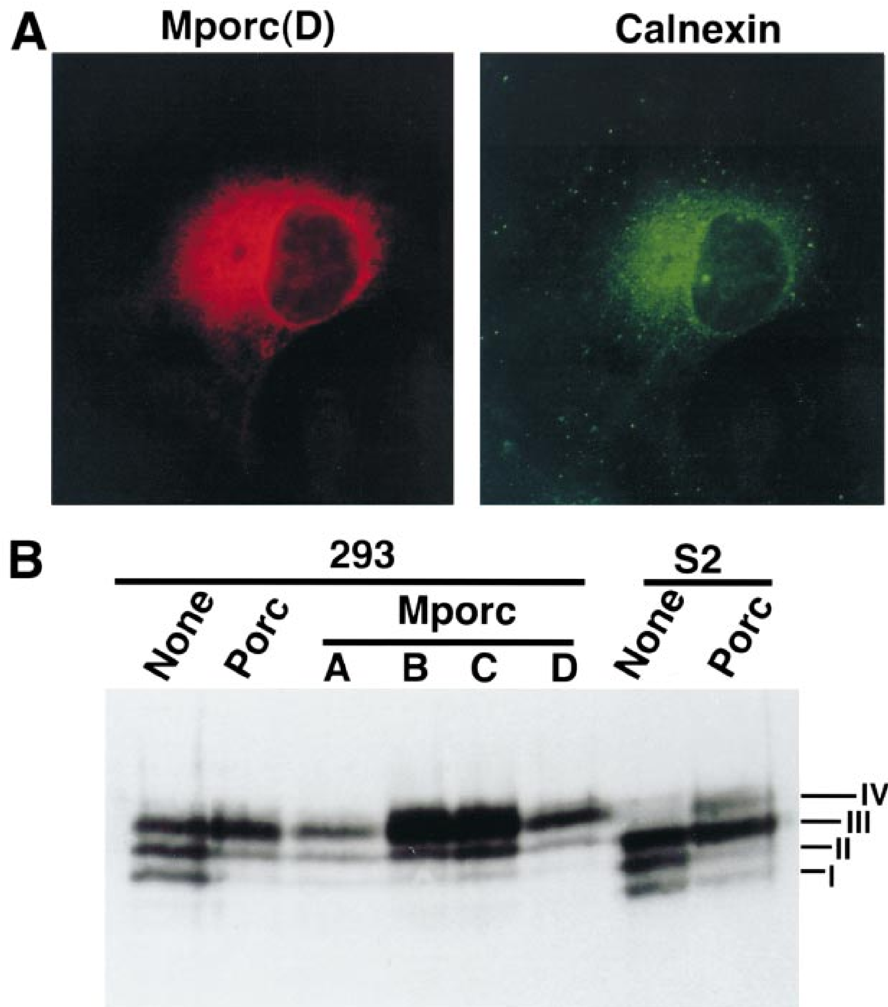


Fig. 6. Intracellular localization of Mporc and the effect of Mporc on the processing of Wg in mammalian cells. (A) Staining pattern of HA-epitope-tagged Mporc (D) expressed in COS-1 cells corresponds to that of calnexin, indicating that Mporc (D) is localized at the ER like Porc in *Drosophila* cells. The other Mporc types also localize at the ER (not shown). (B) The effect of Mporc (A–D) on the processing of Wg expressed in human 293 cells was analyzed by Western blot. As previously reported [15], there are multiple bands with different degrees of N-glycosylation. Form I is the signal-sequence-cleaved but unglycosylated Wg. Forms II, III, and IV have one, two, and three *N*-glycan chains, respectively (see text for details). Equal amounts of forms II, III, and IV are present in the cells transfected with *wg* alone. In the cells transfected with *wg and* either *porc* or *Mporc* (*A–D*), the ratio of the most glycosylated form, IV, to the others increases. The N-glycosylation pattern of Wg appears to be the same in human and *Drosophila* cells because each form of Wg has the same molecular mass in the two types of cell. Form I (signal-sequence-cleaved but not glycosylated) is not present in 293 cells as a promoter/enhancer driving the expression of *wg* is constitutively active and the protein was analyzed by Western blot at the steady-state level. In contrast, this form is present in S2 cells because *wg* expression is induced by a heat shock promoter.

cells. Form I was absent from the transfected 293 cells because Wg is constitutively expressed by a viral promoter/enhancer on the expression construct and analyzed by Western blot at the steady-state level. In contrast, this form was present in the transfected S2 cells because Wg expression is induced by heat shock. Form I can be detected when Wg is analyzed by pulse-labeling with [^{35}S]methionine and immunoprecipitation in the transfected 293 cells (data not shown).

Mporc binds Wnt proteins and modifies their processing

Porc was found to modify the processing of Wg and DWnt-3 in *Drosophila* embryos and cultured cells ([15]; K. Tanaka and T. Kadowaki, unpublished results). Furthermore, Mporc affected processing of Wg in mammalian cells as described above. We therefore investigated whether Mporc modulated processing of various mouse Wnt proteins expressed in COS-1 cells. Multiple bands with different degrees of N-glycosylation were detected for each Wnt as previously reported [2,3] except for Wnt 5A (Fig. 7A). Differential N-glycosylation of Wnt 1 has previously been documented in detail [27]. Wnt 1 has four potential N-glycosylation sites, and, when expressed in QT6 cells, four discrete bands were detected by [^{35}S]methionine/cysteine labeling and immunoprecipitation. The smallest band co-migrates with mutant Wnt 1, which lacks the hydrophobic N-terminal signal sequence, demonstrating that it represents the signal sequence cleaved, but not glycosylated, Wnt 1. By the same analysis, the Wnt 1 mutant lacking one of three N-glycosylation sites (residues 29, 316, and 359) produced three bands (loss of the slowest migrating band compared with the wild-type). Mutation of the remaining N-glycosylation site (residue 346) did not affect the number of bands produced. Triple mutations at residues 316, 346, and 359 resulted in the synthesis of two protein bands (loss of the two most slowly migrating bands). These results indicate that three out of four potential N-glycosylation sites are in fact glycosylated and the multiple bands represent Wnt 1 with different numbers (1–3) of *N*-glycan chains. Of the four bands of wild-type Wnt 1, the two most slowly migrating ones were secreted outside of the cells. The radioactivity of individual bands was measured, and the ratio of the largest band (indicated with an asterisk in Fig. 7A) to the sum of the other bands in each lane was calculated. On the basis of the above results with Wnt 1, this ratio indicates the processing efficiency of Wnt protein in terms of N-glycosylation. These results are summarized in Fig. 7B. All Mporc types affected the processing of Wnt 1, 3A, 4, 6, and 7B in addition to Wnt 3 and 7A (not shown) by shifting the synthesis toward more glycosylated forms. For example, four bands of Wnt 1 were detected in COS-1 cells as in QT6 cells. In the presence of ectopic Mporc (A–D), the synthesis of the two largest bands (competent to secrete as described above) relative to the other lower bands increased. The ratios determined for Wnt 3A and 7B in the presence of Mporc (A–D) must be less than the actual values because the

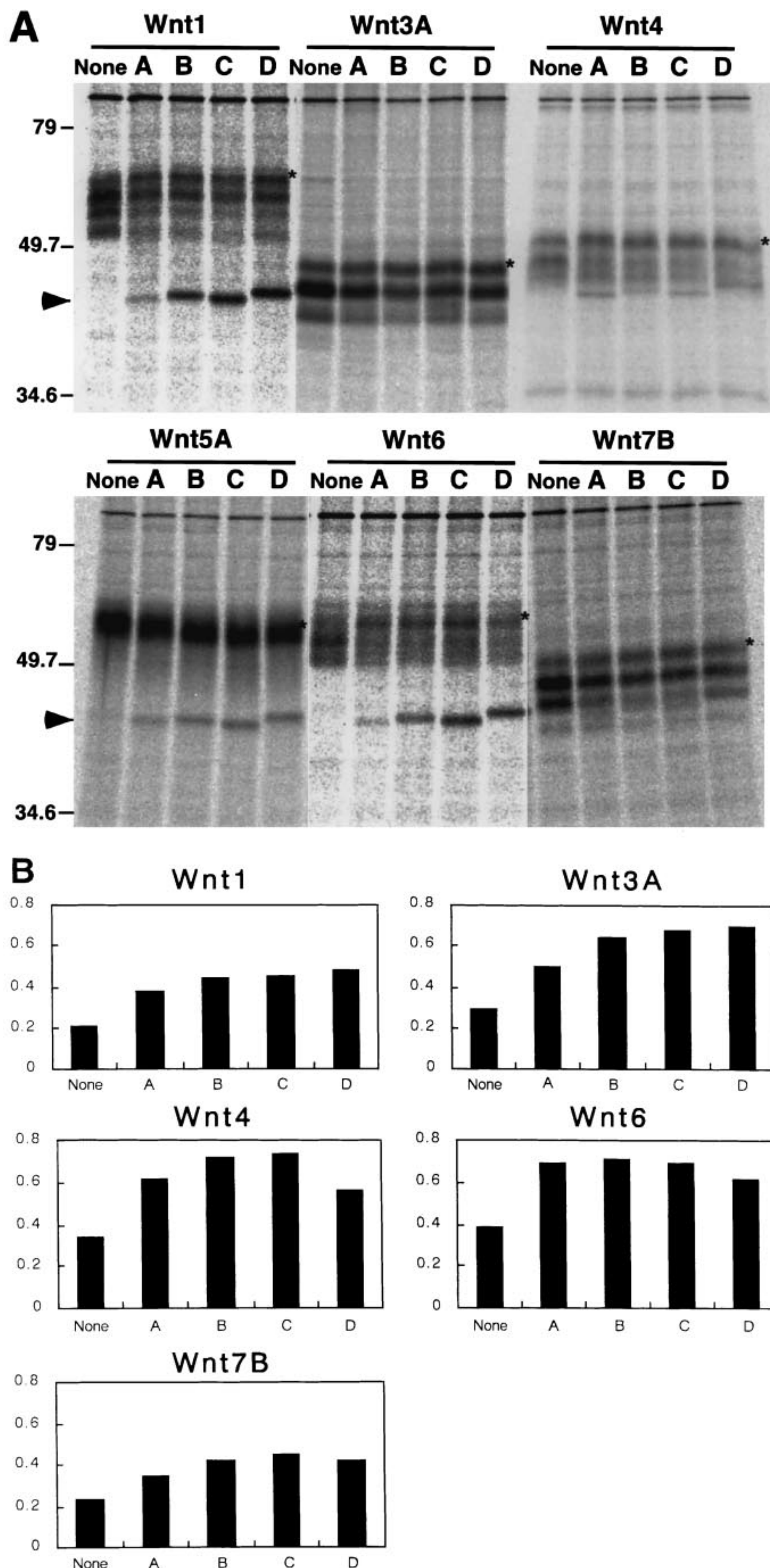


Fig. 7. Effects of Mporc (A–D) on the processing of mouse Wnt proteins. COS-1 cells were transfected with Myc-epitope-tagged Wnts (1, 3A, 4, 5A, 6, and 7B) in the absence (None) and presence of co-transfected Mporc (A–D). The cells were pulse-labeled for 30 min with [^{35}S]methionine and the immunoprecipitated proteins were analyzed by SDS/PAGE (10% gel; A). The molecular sizes of Wnt 1, 5A, and 6 are larger than those of Wnt 3A, 4, and 7B because the former were tagged with triple c-myc epitopes (see Materials and methods). Multiple bands with different degrees of N-glycosylation are detected for each protein except for Wnt 5A (see text for details). The radioactivity of individual bands was measured by a laser image analyzer BAS200 (Fuji) and the ratio of the largest band (shown by an asterisk) to the sum of the others was calculated in each lane (B). Comparing these ratios between None and A–D in each Wnt, all Mporc types enhance the processing of Wnt 1, 3A, 4, 6, and 7B. A single band is detected for Wnt 5A and is not affected by any types of Mporc. The arrowheads in the upper and lower panels of (A) indicate the specific bands co-immunoprecipitated with Wnts in the cells expressing both Wnt and Mporc. These bands are not visible in the Wnt 3A and 7B panels because they co-migrate with these Wnt protein bands. The size of the molecular-mass marker is shown on the left.

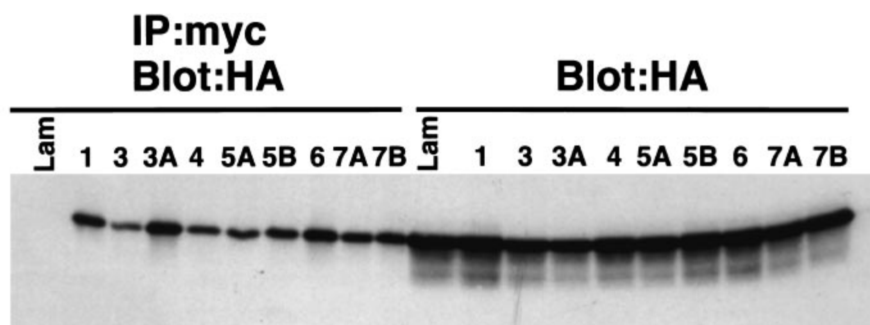


Fig. 8. Co-immunoprecipitation of Mporc (A) and Wnt proteins. COS-1 cells were transfected with the HA-epitope-tagged Mporc (A) and either Myc-epitope-tagged partial laminin β chain (Lam) or Wnt 1, 3, 3A, 4, 5A, 5B, 6, 7A, and 7B. The cell lysates were immunoprecipitated with anti-Myc IgG and then the immunoprecipitates were blotted with mouse anti-HA IgG (left 10 lanes in the panel). Mporc (A) co-immunoprecipitated with all Wnts tested but not laminin β chain. The total cell lysates were also blotted with the same antibody to demonstrate that the same level of Mporc (A) was expressed in all cases (right 10 lanes in the panel). This is an interaction between the membrane protein Mporc on the micelle and the free solubilized secretory protein Wnt.

lower bands of Wnt 3A and 7B overlap with co-immunoprecipitated Mporc proteins (see below). Wnt 5A appears to be efficiently processed in the absence of ectopic Mporc, as a single band was detected in all cases.

Interestingly, one additional band was specifically immunoprecipitated in the cells transfected with both *Wnt* and *Mporc* (shown by arrowheads in Fig. 7). The migration of each band was slightly different and matched the expected molecular mass of Mporc (A–D). To prove that this band represents Mporc, COS-1 cells were transfected with the Myc-epitope-tagged *Wnt 1–7B* and the HA-epitope-tagged *Mporc* (*A*). As a control, a Myc-epitope-tagged partial laminin (a glycosylated cysteine-rich basement membrane protein) β chain [23] was also transfected. The cell lysates were immunoprecipitated with rabbit anti-Myc IgG, and the immunoprecipitates then blotted with mouse anti-HA IgG. Mporc (A) co-immunoprecipitated with all Wnt proteins tested but not laminin β chain (Fig. 8). Thus, Mporc (A–D) bind to Wnt proteins and affect their processing.

DISCUSSION

Human MG61 was first identified as a vertebrate homolog of *porc* through a database search. The MG61 cDNA was used to screen mouse and *Xenopus* homologs of *porc* by low-stringency hybridization. As Porc is involved in the processing of Wg, vertebrate Porc homologs are also expected to have roles in the processing of Wnt, which includes, for example, 17 members in mouse. Thus, multiple genes encoding mouse *porc* homologs with different target specificity may therefore exist. In fact, three mouse genes encoding *dishevelled* homologs (*dvl-1–3*) have been identified even though a single gene exists in *Drosophila* [28–30]. However, this does not seem to be the case for *porc.* From the above screening, only cDNAs representing *Mporc* (*D*) and *Xporc* (*A*) with significant similarity to both MG61 and *porc* were isolated. Furthermore, no other *porc*-related cDNA has been identified in an EST database to date.

The amino-acid sequences of MG61, Mporc, and Xporc are well conserved with a similarity of more than 80%. High similarity is found throughout the entire sequence, including the putative transmembrane domains, suggesting that they may have roles other than as membrane anchors. For example, they may associate with other membrane proteins, or form a channel/pore-like structure in the membrane. Sequence comparison of the vertebrate Porc homologs with other proteins in a database did not reveal any functional domains, suggesting that Porc is a novel family of proteins with specific functions. The hydrophobicity plots of five Porc family members indicate that they share the same overall structure with transmembrane domains at fixed positions. None have the signal sequences therefore the first transmembrane domains appear to act as ER-targeting signals (revealed by the ER localization of the HA-epitope-tagged Mporc at the N-terminus).

As described above, we identified a single gene encoding the mouse *porc* homolog through cDNA and genomic DNA screening. Meanwhile, four types of *Mporc* mRNA were found to be generated by alternative splicing. Similarly, four types of *Xporc* mRNA were identified. These data suggest that the generation of four types of mRNA from a single *porc* gene by alternative splicing is conserved in vertebrates. As only one type of mRNA (corresponding to type *A* of vertebrate *porc*) appears to be produced from *Drosophila porc* and *C. elegans mom-1,* four vertebrate Porc proteins are probably involved in the processing of Wnt (or other proteins) specific to vertebrates. As a result of alternative splicing, a stretch of hydrophilic amino acids after the fifth transmembrane domain in the middle of Mporc and Xporc is affected (indicated by an arrow in Fig. 1B). This is the N-terminal part of the largest domain facing the ER lumen (indicated by a bar in Fig. 1B). Because no difference in the activities of Mporc (A–D) was found when tested against Wnt proteins, it was not possible to demonstrate the significance of the alternative splicing. Mporc (A–D) may have different activities on other Wnt proteins not tested in this study or protein family members other than Wnt. Alternatively, the domain affected by alternative splicing may be important for protein stability in the different types of cell and this effect may be nullified if overexpressed in the transfected cells.

All Mporc types bind various Wnt proteins and affect their processing. None of the Mporc types modified the processing of Wnt 5A (Fig. 7) and 5B (not shown), which have four and five potential N-glycosylation sites, respectively. It is possible that they require less Porc than other Wnts and the endogenous Porc in COS-1 cells is sufficient to support their processing. However, it cannot be ruled out that Mporc modifies the processing of Wnt 5A and 5B other than at the N-glycosylation event. Mporc is identified as another example of a Wnt-binding protein in addition to BiP [4] in the ER. As Mporc does not bind laminin β chain [a glycosylated cysteine-rich basement

membrane protein (Fig. 8)], it must have binding specificity. Whereas the role of BiP in Wnt biogenesis is not known, Mporc binds Wnt proteins in the ER and affects their processing. Thus, Mporc appears to be the first chaperone-like molecule found for Wnt.

Although the Porc family modulates the processing of Wnt proteins, its overexpression alone does not change the total amount of these proteins secreted outside of the cells (not shown). This is probably because other proteins are also necessary for the processing and/or secretion of Wnts. Screening of the *Drosophila* genes involved in Wg signaling identified one that affects Wg processing/secretion besides *porc* [31]. *C. elegans* Mom-3 was also shown to be essential for Wnt signaling in its producing cells in addition to Mom-1 (Porc) [18]. Thus, the Wnt family apparently requires multiple protein factors for its processing and secretion. Mporc is primarily localized at the ER when expressed as the epitope-tagged protein in the transfected cells. *Drosophila porc* embryos can be rescued by injection of *porc* or *Mporc* (to a lesser extent) RNA. Consistent with these data, both Porc and Mporc modify the processing of Wg expressed in mammalian cells. These results demonstrate that Porc family members are evolutionarily conserved ER membrane proteins and bind Wnt family members to modulate their processing.

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