Vertebrate Proteins Related to Drosophila Naked Cuticle Bind Dishevelled and Antagonize Wnt Signaling

Keith A. Wharton, Jr.,*†‡,1 Gregor Zimmermann,*† Raphaël Rousset,*† and Matthew P. Scott*†,2

*Department of Developmental Biology, †Department of Genetics, and ‡Department of Pathology, Howard Hughes Medical Institute, Beckman Center, B300, 279 Campus Drive, Stanford School of Medicine, Stanford, California 94305

Wnt signals control cell fate decisions and orchestrate cell behavior in metazoan animals. In the fruit fly Drosophila, embryos defective in signaling mediated by the Wnt protein Wingless (Wg) exhibit severe segmentation defects. The Drosophila segment polarity gene naked cuticle (nkd) encodes an EF hand protein that regulates early Wg activity by acting as an inducible antagonist. Nkd antagonizes Wg via a direct interaction with the Wnt signaling component Dishevelled (Dsh). Here we describe two mouse and human proteins, Nkd1 and Nkd2, related to fly Nkd. The most conserved region among the fly and vertebrate proteins, the EFX domain, includes the putative EF hand and flanking sequences. EFX corresponds to a minimal domain required for fly or vertebrate Nkd to interact with the basic/PDZ domains of fly Dsh or vertebrate Dvl proteins in the yeast two-hybrid assay. During mouse development, nkd1 and nkd2 are expressed in multiple tissues in partially overlapping, gradient-like patterns, some of which correlate with known patterns of Wnt activity. Mouse Nkd1 can block Wnt1-mediated, but not β-catenin-mediated, activation of a Wnt-dependent reporter construct in mammalian cell culture. Misexpression of mouse nkd1 in Drosophila antagonizes Wg function. The data suggest that the vertebrate Nkd-related proteins, similar to their fly counterpart, may act as inducible antagonists of Wnt signals. © 2001 Academic Press

Key Words: naked cuticle; Nkd; wingless; Wnt; dishevelled; dsh; Dvl; development; signal transduction; Drosophila; mouse; human; inducible antagonist.

INTRODUCTION

A majority of genes essential for developmental patterning in the fruit fly, Drosophila melanogaster, have vertebrate counterparts. The segment polarity genes, which encode signal transduction components for the secreted molecules Wingless (Wg, a Wnt protein) and Hedgehog (Hh), are prime examples. Early in embryonic segmentation, each signaling protein maintains the production of the other in adjacent cells, forming a positive regulatory loop (DiNardo et al., 1994). Later in development, the two signaling pathways are repeatedly used in patterning and induction events. Similarly, Wg and Hh homologs are essential for a vast range of signaling events during vertebrate development. Both pathways have been linked to human cancer, particularly colon, breast, and epidermal appendage tumors for the Wnt pathway (Morin, 1999) and basal cell carcinoma and medulloblastoma for the Hh pathway (Goodrich and Scott, 1998).

Many segment polarity genes, such as dishevelled (dsh) for the Wnt pathway, were first discovered in flies and subsequently isolated in vertebrates (Klingensmith et al., 1996). Other Wnt pathway components were first discovered in other organisms and later found in the fly. For example, the first vertebrate Wnt gene, Wnt1, was isolated as a mouse oncogene (Usse et al., 1984), while β-catenin, a homolog of fly Armadillo (Arm), was initially described as a cell adhesion molecule-associated protein (Ozawa et al., 1989; Peifer et al., 1992). The similarities between fly
Segment polarity genes and their vertebrate counterparts have helped elucidate common signal transduction mechanisms and have greatly aided in the understanding of both developmental biology and cancer.

Eighteen Wnt proteins have been found in both the mouse and the human genomes (see Wnt homepage http://www.stanford.edu/~rnusse/wntwindow.html). In the developing mouse Wnt signals are typically produced in partially redundant and highly dynamic patterns throughout embryogenesis. Mutations in a few Wnt genes have

**FIG. 1.** Drosophila and vertebrate Nkd proteins share the EFX domain. (A) Schematic alignments of Recoverin (Rec, black), Drosophila Nkd (DNkd, yellow), and the mouse (m) and human (h) Nkd-related proteins Nkd1 (orange) and Nkd2 (green). Recoverin has four EF hands, numbered 1–4. DNkd shares sequence similarity with the third EF hand of Recoverin (3) (Zeng et al., 2000). DNkd shares two regions of similarity with the four vertebrate Nkd-related proteins: a histidine-rich C-terminus (H), and the EFX domain. The N-terminus of all four vertebrate proteins has a myristoyl consensus (horizontal stipple). mNkd1 has a rare alternatively spliced form without a myristoyl consensus (vertical stipple). Linewidth designates length of 100 amino acids. Sequence alignment below shows encoded amino acid sequences in DNkd and the four vertebrate Nkd proteins; identical amino acids are in black box, while conservative changes are in gray. The consensus EFX residues (EFX cons) are aligned with the consensus EF hand residues below (EF cons). Key: capital letters, standard amino acid codes; h, hydrophobic residue; b, basic residue; a, acidic residue; O, oxygen-donating residue in EF hand; *, variable residue; J, hydrophobic residue; X, Y, Z, residue that coordinates ion binding in x, y, and z axes. (B, C) Adult multiple-tissue Northern blot hybridized to mouse nkd1 (B) or mouse nkd2 (C) probe. Transcript size in kilobases (kb) is designated to the right of each blot. Key: B, brain; H, heart; K, kidney; L, lung; T, testis; S, skin.
FIG. 2. Encoded amino acid sequences of vertebrate Nkd proteins. Boxshade alignment of the four vertebrate Nkd proteins. Residues identical in all four encoded proteins are in black box and conservative changes are in gray. Nkd1-specific residues are orange, and Nkd2-specific residues are green. Red bar shows the EFX domain. Consensus residues are designated as in Fig. 1. The cDNA sequences have been deposited to Genbank under the following Accession Nos.: mNkd1, AF358134; mNkd2, AF358136; hNkd1, AF358135; hNkd2, AF358137.
revealed their importance for murine development. For example, Wnt1 is required for midbrain/hindbrain formation (McMahon and Bradley, 1990), Wnt3 for early gastrulation (Liu et al., 1999), Wnt3A for tailbud development (Takada et al., 1994), Wnt4 for renal development (Stark et al., 1994), and Wnt7A for dorsal limb bud development (Parr and McMahon, 1995). In addition, mutations affecting the Wnt signal transduction components β-catenin, APC, or Axin that result in the accumulation of β-catenin can cause cancer in mice and humans (Polakis, 1997; Rubinfeld et al., 1997; Satoh et al., 2000).

Insights into the complexity of Wnt signal regulation have been gleaned from studies in model genetic organisms such as the nematode and fruit fly. Genetic analysis in the fly originally ordered the functions of a handful of segment polarity genes required for Wg signal transduction. In cells that send the signal, porcupine (porc) is required for Wg secretion (Kadowaki et al., 1996). In responsive cells the Wg signal is transmitted sequentially through the action of dsh, zeste-white 3 (zw3), and arm (Nordermeer et al., 1994; Siegfried et al., 1994). Similar genetic experiments in the nematode confirmed and extended the fly epistasis results (Han, 1997). Recently the number of protein families implicated in Wnt signaling has grown from 5 to over 40 (Wnt interaction network). A common target of Wnt signaling is Armadillo/β-catenin (Peifer et al., 1992, 1991). In the absence of a Wnt signal, β-catenin undergoes phosphorylation-dependent ubiquitination and rapid degradation by proteasomes (Aberle et al., 1997). Wnt signaling antagonizes this phosphorylation, resulting in β-catenin accumulation (Peifer et al., 1994) and nuclear translocation (Schneider et al., 1996). Once in the nucleus, β-catenin binds TCF/LeF family DNA-binding proteins to directly regulate many Wnt-responsive target genes (Behrens et al., 1996; Brannon et al., 1997; Molenaar et al., 1996; Riese et al., 1997). However, some Wnt proteins can act independently of β-catenin (Kengaku et al., 1998), or even independently of transcription, to promote Ca2+ fluxes (Slusarski et al., 1997), control cell polarity (Han, 1997), or regulate cell shape changes (Heisenberg et al., 2000).

Because excess Wnt signaling can initiate unregulated cell proliferation, it is critically important to understand how Wnt signals are normally regulated during development and throughout the life of the organism. In principle, proteins that regulate signal transduction can act extracellularly, in the cytoplasm, or in the nucleus. In Wnt signaling, families of secreted or membrane-bound proteins present in the extracellular space, including FrzB, Cerberus, Dickkopf, and Wif, as well as proteoglycans, regulate Wnt distribution (Pfeiffer and Vincent, 1999). In the nucleus, Groucho acts as transcriptional co-repressor with TCF/LeF in the absence of early Wg signals (Cavallo et al., 1998).

A growing body of evidence suggests that developmental signaling is autoregulated via positive and negative feedback loops (Perrimon and McMahon, 1999; Freeman, 2000). We recently showed that naked cuticle (nkld), originally isolated in the pioneering genetic screens of Nüsslein-Volhard and Wieschaus (Jürgens et al., 1984), acts in a negative feedback loop with Wg (Zeng et al., 2000). nkld was one of the last segment polarity genes to be cloned and encodes a cytoplasmic Wg-inducible protein that can bind to and antagonize the function of Dsh (Rousset et al., 2001; Zeng et al., 2000). This elegant control system, essential for limiting the early effects of Wg in Drosophila segmentation, allows the Nkd antagonist to accumulate in proportion to Wg signal strength or duration. In this paper, we describe two mouse and human genes related to Drosophila nkld. Our data suggest that these genes, similar to their fly counterpart, may act as inducible antagonists for vertebrate Wnt signals.

**MATERIALS AND METHODS**

**Cloning of nkld-Related Genes**

Mouse Nkd1, identified by sequence similarity to fly Nkd, was originally found as expressed sequence tag (EST) W78547. The insert from this EST was used as a probe to screen λ-phage cDNA libraries from 8.5 d.p.c. mouse embryos (generously provided by Brigid Hogan) and adult mouse lung (Stratagene), and the clones harboring the entire open reading frame (L84 and L85) were sequenced. A single phage clone retrieved twice (L16, L79) from screening 2 × 106 phage clones in the 8.5 d.p.c. library encoded an alternate 5' end that was spliced in frame with amino acid 8 of mNkd1 and contained almost the entire remainder of the mNkd1 coding region. These unique sequences are also present on a mouse bacterial artificial chromosome clone encoding nkld1 (K. A. Wharton, unpublished data), suggesting that this represents a rare splice variant and not a chimeric clone.

Mouse Nkd2 was originally identified as EST AA756769 and AA794228 because of their similarity to mNkd1 downstream of the EF hand. λ-phage screens revealed two clones with alternate 3' untranslated regions (L2-1 and L2-5). Each clone was sequenced, and ESTs now exist that extend these clones 3' and 5'. The remainder of the 5' sequence of mouse nkld2 was retrieved from EST clone BE291907, which represents a partially spliced clone.

Human nkld1 was originally identified from EST H55148 and subsequently from genomic scaffold sequence present on high-throughput genome sequence (HTGS) clones AC007608 and AC007334. Human nkld2 was originally identified from EST AA101288 and subsequently in HTGS clones AC010446 and AC016498. The human sequences in Fig. 2 were assembled using a combination of EST and genomic clone sequence based on their high degree of similarity to the mouse sequences in coding regions and near-conserved splice junctions (not shown).

Northern analysis was performed using hybridization conditions specified by the manufacturer on a multiple tissue blots from Stratagene and Origene (Fig. 2). A GAPDH probe was hybridized to the blot to ensure equal loading in all lanes (not shown).

The sequence alignments were made using Clustal and linked Boxshade utilities that are available at http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html.
Yeast Two-Hybrid and Binding Assays

Studies were performed as previously described (Rousset et al., 2001). For the yeast two-hybrid assay, the bait plasmid was pAS2-1 and the prey plasmid was pACT2 (Clontech). The Nkd-EFX plasmids correspond to amino acids 177–253 of DNkd, 125–190 of mNkd1, and 121–186 of mNkd2. The Dsh-basic/PDZ plasmids correspond to amino acids 167–338 of Dsh, 163–337 of mDvl1, 180–353 of mDvl2, and 161–335 of mDvl3 (all homologous regions based on Clustal alignments.) The transformation of yeast strain PJ69-4A (containing the ADE reporter gene) was achieved using a variation of the lithium acetate method (Clontech). Yeast growth on media ± adenine was compared after 5–6 days at 30°C. We did not test the Dsh/Dvl sequences in the bait plasmids because Dsh activates transcription by itself when present in the bait plasmid (Rousset et al., 2001).

For the GST pulldown assay, full-length DNkd and mNkd1 proteins were produced and labeled with 35S-methionine using the TNT T7-coupled reticulocyte lysate system (Promega) from pBlue-script IIKS(+) derived constructs. The GST-Dsh plasmid has been described previously (Willert et al., 1997).

Whole-Mount In Situ Hybridization

Embryos were harvested from pregnant C57BL6 female mice and immediately fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS). The entire procedure was performed as previously described (Goodrich et al., 1996). Embryos were photographed under darkfield or brightfield on a Leica M10 stereomicroscope. Sense and antisense probes were synthesized with comparable specific incorporation of digoxigenin-conjugated nucleotide, and the sense probes did not show any background staining (not shown).

Section In Situ Hybridization

Paraffin sections were rehydrated through graded alcohols and washed in PBS. Frozen and paraffin sections were hybridized with the digoxigenin-labeled probes overnight at 60°C and washed in 50% formamide, 2× SSC at 60°C, and then were incubated with alkaline phosphatase-conjugated antidigoxigenin antibody (Roche) overnight at 4°C. Sections were washed with PBS and visualized with BM Purple-AP substrate (Roche). The slides were dehydrated with 95% ethanol, counterstained with Eosin Y (Fig. 5C), and photographed with a blue filter on a Zeiss Axioskop.

Wnt-1 Reporter Assay

Cos7 or HEK293T cells were cultured in DMEM containing 10% fetal calf serum, 100 u/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamate, and 0.1 mM nonessential amino acids. Both cell types were transfected at subconfluence with Fugene 6 (Roche) as per manufacturer’s instructions. Wnt1-dependent TopFlash activity was obtained by co-culture of HEK293T cells expressing Wnt1 and Cos7 cells cotransfected with the TopFlash reporter, lacZ expression plasmid (pCDNA-lacZ; a gift of L. Collier), and either mouse nkd1 or empty vector expression plasmids. All cDNA expression vectors employed the cytomegalovirus promoter to drive expression, and mouse nkd1 was cloned into pCDNA as a myc-His6 fusion protein. For each experiment, 2 × 10⁴ HEK293T cells were transfected with 10 μg of the Wnt1 expression plasmid (pECG; a gift of B. Yu and R. Nusse). Cos7 cells (5 × 10⁴) were plated in each well of a 24-well plate and transfected with 0.02 μg TopFlash TCF luciferase reporter construct (Upstate Biotechnology), 0.02 μg pCDNA-lacZ, and the indicated amounts of pCDNA-mNkd1mycHis6. After 6–8 h, both cultures were washed 3 times with phosphate-buffered saline, and then returned to the incubator. The luciferase assay was performed 12 h later on a luminometer using the Dual-Light Reporter Gene Assay System (Perkin-Elmer, as per manufacturer’s instructions). Data was normalized for transfection efficiency based on lacZ expression. The total amount of DNA was kept constant for all transfections in order to obtain comparable trans-
fection efficiencies. β-catenin-induced activity was performed similarly, except that instead of the Wnt1 expression plasmid, 0.04 μg of β-catenin expression plasmid (pRK5SK; a gift of B. Yu and R. Nusse) was transfected directly into the Cos-7 cells in each well.

**Misexpression of mNkd1 in the Fly**

Myc-tagged (clone L16) and untagged (clone L84) versions of mouse nkd1 were cloned into pUAS-T and transformed into w or yw flies using standard methods as previously described (Zeng et al., 2000). Similar phenotypes were obtained using tagged and untagged constructs in multiple independent transformant lines. All crosses were performed at 29°C. E105-Gal4 and B119-Gal4 have been previously described (Zeng et al., 2000).

**RESULTS**

**Identification of nkd-Related Genes**

Searches of public expressed sequence tag databases revealed mouse and human EF hand-encoding cDNA sequences related to fly nkd (Fig. 1A and Materials and Methods). Subsequent phage library and public database screens revealed two nkd-related genes each in mouse (m) and human (h), here called nkd1 and nkd2 (Fig. 2).

Comparison of the fly Nkd amino acid sequence with those of the vertebrate Nkd-related proteins reveals two regions of similarity. First, both fly and vertebrate Nkd proteins share a histidine-rich C-terminus (7/10 amino acids in the fly protein, 12/15 and 16/19 amino acids in vertebrate Nkd1 and Nkd2 proteins). While His-rich regions have been described in many proteins, the His repeats at the C-terminus of the vertebrate Nkd proteins are interrupted by conserved glutamate residues (Fig. 2). Second, the fly and vertebrate proteins share an approximately 66 amino acid region of similarity including the putative EF hand. We term this region the EFX domain (Fig. 1A). The amino acid sequences of the EFX domains are 42% identical between fly Nkd and mNkd1, and 41% identical between fly Nkd and mNkd2.

EF hands are modular, helix-loop-helix Ca^{2+} binding

**FIG. 4.** nkd1 and nkd2 expression patterns during mouse embryogenesis. Whole-mount in situ hybridization to 9.5 days postcoitum (d.p.c.) (A–D), 10.5 d.p.c. (E, F), and (G–J) 12.5 d.p.c. mouse embryos stained for nkd1 (A, C, E, G, I) or nkd2 (B, D, F, H, J). (A, B) Right lateral view with head up. Red arrowheads designate presence (A) or absence (B) of dorsal CNS staining. (C, D) Higher power of lower part of embryos in A and B showing similar forelimb (fl) and somite

(s) expression. Red arrowhead designates dorsal CNS staining in C that is absent in D. (E, F) Right lateral view of trunk showing dorsal CNS stain in E (red arrowheads) that is absent in F. Blue arrowhead highlights tailbud expression that is similar in nkd1 and nkd2. (G, H) Right (G) and left (H) lateral views of bisected head showing generalized subepidermal mesenchyme expression that is downregulated in primitive whisker follicles (blue arrowhead). As whisker follicle developments proceed mediolaterally, more mature medially placed follicles express nkd1 (red arrowheads) but not nkd2. (I, J) Median sagittal view of bisected embryos (dorsal up) showing expression of nkd1 (I) but not nkd2 (J) in Rathke's pouch (red arrowhead), but similar expression in the subepidermal mesenchyme of the snout, soft palate, tongue (t), and mandible (blue arrowhead).

*98* Wharton et al. Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.
motifs present in many Ca\textsuperscript{2+} binding proteins, including calmodulin and recoverin (Kawasaki et al., 1998). EF hand-containing proteins typically have between two and four consecutive EF hands, but proteins with single EF hands have been described (Mochizuki et al., 1996). Previous sequence comparisons suggested that, among published protein sequences, *Drosophila* Nkd shared the greatest sequence similarity with the high-affinity Ca\textsuperscript{2+} binding EF hand (EF3) of the recoverin subfamily of EF-hand proteins (Zeng et al., 2000). Recoverins are N-terminally myristoylated, Ca\textsuperscript{2+}-dependent switch proteins that regulate the localization and activity of a variety of enzymes from yeast to mammals (Burgoyne and Weiss, 2001). The EF hand region of mNkd1 is 35% identical over 53 amino acids to *Drosophila* Frequenin (Pongs et al., 1993), the recoverin subfamily EF hand protein most closely related to mNkd1.

**FIG. 5.** Fly and vertebrate nkd gene expression patterns. (A) Whole-mount in situ hybridization of nkd in the third instar *Drosophila* wing pouch shows wing margin expression (large arrowhead) tapering off in a gradient on either side of the wing margin (small arrowheads). (B) Whole-mount in situ hybridization of 11.5 d.p.c. mouse embryo showing gradient-like expression of nkd1 in branchial arch and face elements. Eye placode is in the center. Highest expression is seen at arch boundaries (large arrowhead) with expression tapering off away from the boundaries (small arrowhead). (C, D) Similar gradient expression of mNkd in snout mesenchyme (C) in P0 (newborn) mouse skin and perichondrium (D) in 15.5 d.p.c. hindlimb revealed by section in situ hybridization. Note how expression peaks at epidermal/mesenchyme (C) or cartilage/perichondrium (D) boundary (large arrowhead) and tapers away in the adjacent mesenchyme (small arrowhead). wf, whisker follicle; lc, longbone cartilaginous condensation. (E) Side view of 10.5 d.p.c. embryo forelimb stained with nkd1 showing greater expression in dorsal (D) than ventral (V) limb mesenchyme. nkd2 does not display this bias during this stage (not shown). Arrowhead, position of apical epidermal ridge.
(not shown). While the Drosophila Nkd EFX is about 40% identical over 66 amino acids to all of the vertebrate EFX sequences, the vertebrate EFX sequences are all 84% identical to each other over the same region (Fig. 1A). In some characterized EF hand motifs, amino acid substitutions have occurred that preclude ion binding, indicating those domains do not serve as ion sensors. Both fly and vertebrate nkd sequences encode oxygen-donating residues in EF loop positions known to coordinate ion binding (Fig. 1A), although fly Nkd has an unusual pair of histidine residues at the apex of the loop that could, based on comparisons with EF hand-crystal structures, alter ion binding. We do not know whether any of the Nkd proteins bind Ca\textsuperscript{2+} or indeed whether they adopt standard EF hand conformations.

Examination of all four vertebrate Nkd-related protein sequences (Fig. 2) reveals that all four vertebrate genes form a gene family with 36% overall amino acid identity. The vertebrate proteins share blocks of amino acid conservation on either side of the EF hand that are not obviously shared with fly Nkd (Fig. 2, and not shown). Supporting the idea that nkd1 and nkd2 represent distinct orthologs, the amino acid sequence of mNkd1 is 86% identical to hNkd1, but only 43% identical to hNkd2. Conversely, mNkd2 is only 43% identical to hNkd1 but 75% identical to hNkd2. Further, mNkd1 and hNkd1 have identical amino acids in 206/309 positions (66%; orange residues in Fig. 2) that are different in mNkd2 and hNkd2, excluding amino acid residues shared among all four encoded proteins. Similarly, mNkd2 and hNkd2 have identical amino acids in 164/309 positions (53%; green residues in Fig. 2) that are different in mNkd1 and hNkd1. All four Nkd-related proteins, similar to the recoverins, have a myristoylation consensus at their N-terminus (MGKxxSK), although we do not know whether the vertebrate Nkd-related proteins are indeed myristoylated. In contrast, fly Nkd does not have an N-terminal myristoyl consensus sequence (Zeng et al., 2000). According to high-throughput genome sequence mapping data, human nkd1 maps to 16q12, and human nkd2 maps to 5p15.3, the latter of which we confirmed by radiation hybrid analysis (not shown).

**Nkd EFX Domains Bind Dishevelled Basic/PDZ Domains**

We recently showed that in Drosophila, Nkd targets the Wnt signaling component Dishevelled (Dsh) via a direct interaction with the Dsh basic and PDZ (basic/PDZ) domains (Rousset et al., 2001). If the Nkd/Dsh interaction has been important for the control of Wnt signaling during evolution, then the region in Drosophila Nkd that binds Dsh should be conserved in a vertebrate Nkd and bind the vertebrate Dsh homologs, Dvl1, Dvl2, and Dvl3. Yeast two-hybrid studies showed that the EFX domains of fly and mouse Nkd proteins interacted with the basic/PDZ domains of fly and mouse Dsh proteins (Fig. 3A). The mNkd2-EFX was less effective than mNkd1-EFX at interacting with the Dvl proteins in this assay. This is unlikely to be due to

![FIG. 6.](image-url) Nkd blocks Wnt1-induced, but not β-catenin-induced, transcription in cultured cells. Luciferase activity was determined after Cos7 cells transfected with the TopFlash Luciferase reporter were incubated with Wnt1-expressing HEK293T cells (Wnt1) or when the Cos7 cells were cotransfected with β-catenin (B). The effect of increasing amounts of cotransfected mNkd1 plasmid (μg mNkd1) into Cos7 cells was evaluated for both activators. The level of Wnt1 or β-catenin-dependent activation was set at 100% in the presence of Wnt1 or β-catenin in the absence of mNkd1, and is 5 to 7 times the level of activity seen in the absence of Wnt1, β-catenin, and mNkd1 (Basal). (A) Expression of increasing amounts of mNkd1 results in a dose-dependent block in luciferase activity to baseline levels. (B) Expression of comparable amounts of mNkd1 does not block β-catenin-induced activation of luciferase activity. For both graphs, data are expressed as normalized luciferase activity. Assays were performed in triplicate (bars = standard deviation) and are representative of at least three independent experiments.
In 8.5 days postcoitum (d.p.c.) mouse embryos, nkd1 expression is observed throughout the entire rostrocaudal axis (not shown). By 9.5 d.p.c., nkd1 and nkd2 transcripts accumulate in the anterior and posterior of each somite boundary (Figs. 4C and 4D). Branchial arch expression is evident by 9.5 d.p.c. and is well developed for both genes by 10.5 d.p.c. (Fig. 5B and not shown). In later embryos, nkd1 is transcribed in the endocardial cushion, pulmonary epithelium, renal mesenchyme, hair follicles, and in other tissues (Fig. 5 and not shown). The mouse nkd expression patterns present three striking features. First, nkd1 and nkd2 are expressed in a partially overlapping fashion, with nkd1 generally being expressed more broadly (or at higher levels) than nkd2. Second, in many cases nkd1 and/or nkd2 transcripts are distributed in a gradient emanating from a tissue boundary, such as the boundaries between branchial arches (Fig. 5B), skin epithelium and mesenchyme (Fig. 5C), and the somites (Figs. 4C and 4D). Third, there is some overlap between the distribution of the nkd genes and Wnt genes in places where Wnt functions have been well-studied. For example, Wnt1, Wnt3, and Wnt3A are all expressed in the mouse dorsal neural tube and serve partially redundant roles in neural development (Ikeya et al., 1997; Parr et al., 1993). nkd1, but not nkd2, RNA is highly enriched in the dorsal neural tube (Figs. 4A, 4C, and 4E) and extends in a gradient into the ventricular zone (not shown). Both nkd1 and nkd2 RNAs are expressed in the tailbud (Figs. 4E and 4F), where Wnt3A is expressed and plays an essential role in development (Takada et al., 1994). In 10.5 d.p.c. embryos, nkd1 RNA is present in dorsal limb bud mesenchyme, which is the tissue targeted by a β-catenin-independent dorsalization signal mediated by Wnt7A (Kengaku et al., 1998; Riddle et al., 1995). In Drosophila, we noted a striking correlation between nkd and Wg distribution, in a pattern that mimics the Wg activity gradient (Zeng et al., 2000) (Fig. 5A). Similarly, in mouse tissues, some gradient patterns of nkd expression bear a striking resemblance to the distribution of known Wnt activities.
adults that were missing hemisternite bristle clusters (Fig. 7A) or had laterally displaced sternite bristles (not shown). This phenotype is indicative of compromised wg signaling during metamorphosis (Shirras and Couso, 1996) and is also seen in otherwise wild-type adults that have been exposed to low-level overexpression of fly Nkd (Zeng et al., 2000).

**FIG. 7.** Misexpression of mNkd1 in Drosophila results in wg-like adult phenotypes. (A) Ventral abdomen of B119-Gal4; UAS-mNkd1 adult female showing segmentally repeated sternite bristle clusters. One segment shows loss of a hemisternite bristle cluster (yellow arrow) and lateral displacement and disorientation of the contralateral bristle cluster (green arrow). (B) Ventral abdomen of typical wg, B119-Gal4; UAS-mNkd1 adult female showing consistent loss of some bristles in every segment with laterally displaced (green arrow) and missing (yellow arrows) hemisternite bristle clusters. (C) Wild-type leg. (D) Representative legs from wg, E105-Gal4; UAS-mNkd1 adults are variably truncated.
The effect of overproduced fly Nkd is enhanced by loss of a wild-type copy of a positively acting component of Wg signaling such as wg, dsh, or arm (Zeng et al., 2000). We therefore asked whether mNkd1 would be more potent in flies compromised for Wg signaling. We used two Gal4 driver lines, B119 and E105, that are effective lines for revealing low-level antagonism of Wg activity by mutant forms of fly Nkd (K. A. Wharton, unpublished data). Production of mNkd1 using B119-Gal4 in heterozygous wg/+ animals consistently resulted in loss of hemisternite bristle clusters (Fig. 7B). Some adults completely lacked sternite bristles (not shown), a phenotype similar to that seen when fly Nkd is produced in wild-type animals using the same Gal4 line (Zeng et al., 2000). When fly Nkd was produced using E105-Gal4, most adults had severe leg truncations, another phenotype associated with loss of wg (Zeng et al., 2000). Production of mNkd1 using E105-Gal4 produced rare adults with leg truncations (<1%). In wg+ animals, E105-driven mNkd1 production resulted in up to 12% of adults (n = 158 adults) with at least one truncated leg (Fig. 7D). The mNkd1-induced phenotypes were also enhanced in adult Drosophila heterozygous for a dsh mutation (not shown). Based upon our analysis of the transformations observed with multiple Gal4 lines, the mNkd1-induced phenotypes were qualitatively similar to, but less penetrant and expressive than, those induced by fly Nkd (not shown). Thus, mNkd1, similar to fly Nkd, can antagonize Wnt signaling in both vertebrate and invertebrate systems.

**DISCUSSION**

Our previous studies have shown that Drosophila nkd acts as an inducible antagonist for Wg signaling during early segmentation (Zeng et al., 2000). nkd encodes a novel cytoplasmic protein that acts through the Wnt signaling component Dsh (Zeng et al., 2000; Rousset et al., 2001). The mammalian proteins we identify here appear to be Drosophila nkd orthologs by three criteria. First, the vertebrate Nkd proteins share a conserved region with fly Nkd, the EFX domain, that is necessary and sufficient for the interaction between Nkd and the basic/ PDZ region of Dsh (Rousset et al., 2001; Rousset et al., manuscript in preparation). Second, just as fly nkd is inducible by Wg signals (Zeng et al., 2000), the vertebrate nkd genes are expressed in gradient patterns reminiscent of Wnt signaling gradients. Third, production of one of the mouse Nkd proteins, mNkd1, in cultured cells or flies antagonizes Wnt-dependent readouts. The data are consistent with the idea that these proteins, similar to fly Nkd, are inducible antagonists for vertebrate Wnt activity.

We show that the EFX domain conserved between fly and mouse Nkd proteins corresponds to a minimal domain that interacts with Dsh and the three mouse Dsh homologs, the Dvl proteins. The evolutionary conservation of the binding domain supports the importance of the Nkd/Dsh interaction in Wnt signaling. Nonetheless, there are no apparent nkd homologs in the completed Caenorhabditis elegans genome despite there being five Wnts and at least three Dsh-related proteins (see Wnt homepage). In the current view of metazoan evolution, the common nematode/fly ancestor is thought to be less ancient than the common fly/vertebrate ancestor, implying that C. elegans must have discarded its nkd gene (de Rosa et al., 1999). Five of the common Wnt signaling components were recently described in a primitive metazoan, the Cnidarian Hydra (Hobmayer et al., 2000), so it will be interesting to see whether they too have a nkd gene. One possible explanation for the apparent absence of nkd from C. elegans is that nkd may have evolved to control Wnt signaling over many more cell diameters than are usually employed by Wnt signals in C. elegans. For example, mom-2 is required in the P2 blastomere for spindle orientation and cell fate determination in the immediately adjacent EMS blastomere (Han, 1997). However, lin-44 controls polarized cell divisions over many cell diameters (Herman et al., 1995), and eg-20 controls cell migrations occurring over half of the length of the animal (Wangbo and Kenyon, 1999), so other mechanisms may limit Wnt signaling over many cell diameters in C. elegans. No additional genes closely related to nkd are apparent in the completed D. melanogaster genome. Therefore, redundancy between nkd and another related gene cannot account for the absence of cell-autonomous phenotype in nkd−/− clones in many adult Drosophila tissues (Zeng et al., 2000). One possible explanation for these fly and worm mysteries is that a protein with sequence distinct from Nkd might play similar regulatory role.

The relationship between wg and nkd in the fly may provide some clues to understand how the Nkd-related proteins might regulate Wnt activity vertebrates. In Drosophila embryos, Wg controls the transcription of the target genes en and hh in cells adjacent to the cells that produce Wg (Lee et al., 1992; Martinez Arias et al., 1988; Tabata et al., 1992). In nkd mutants, both en and hh are transcribed in broader stripes than in wild type, at a time when there is no apparent abnormality in Wg protein distribution (Martinez Arias et al., 1988; Moline et al., 1999). One interpretation of the abnormalities in en and hh expression in the nkd embryo is that mutant cells are hypersensitive to Wg (Zeng et al., 2000). By analogy to the fly, a mammal with compromised nkd activity might be hypersensitive to certain Wnt signals. Mammalian Nkd5s may cooperate with or act redundantly with other Wnt pathway antagonists such as Axin and APC. Mouse axin mutants display both recessive lethal and dominant visible phenotypes that suggest excess Wnt signaling: dominant mutants display partial axis duplications, and recessive mutants display ectopic axes (Perry et al., 1995). Homozygous mouse mutants harboring an APC mutation die during early gastrulation (Moser et al., 1995), possibly due to abnormal Wnt signaling. It will be of interest to determine whether mice with mutations in nkd1 and or nkd2 exhibit similar phenotypes.

In some Drosophila tissues Wg is distributed in a gradient (Strigini and Cohen, 2000) and can act as a morphogen, i.e,
in a concentration-dependent fashion, to regulate tissue-specific target genes (Strigini and Cohen, 1999). Wg appears to induce nkd transcription in many tissues and at different stages in embryos and imaginal discs (Zeng et al., 2000). In several tissues nkd is transcribed where the Wg signal has been received, indicating that nkd may be a common target of Wg signaling in the fly. The expression patterns of the mouse nkd genes suggest that the same may be true in mammals. However, not all fly nkd transcription is dependent upon an inducing Wg signal. The initial transcription of nkd in the embryo, for example, probably occurs in response to early segmentation gene activities (Zeng et al., 2000). Similarly, only some of the mouse nkd transcription patterns may be dependent on Wnt signals.

Epistasis experiments employ double mutants to reveal relationships between genes with distinct phenotypes that may act in a common biological pathway. Epistatic relationships are relatively straightforward to determine in the fly and are often applicable to conserved vertebrate pathways. In wgnmnts, in contrast to nkd mutants, en and hh transcription initiates normally but fades during the time when en and hh normally require Wg for maintenance of their expression (Bejsovec and Martinez Arias, 1993; Lee et al., 1992; Martinez Arias et al., 1998; Tabata et al., 1992). Transcription of en in wg nkd double-mutant embryos fades as in wg single mutants (Bejsovec and Wieschaus, 1993), indicating that nkd requires intact Wg signaling to reveal its loss of function phenotype.

One interpretation of the similarity between the wg and wg nkd double-mutant phenotypes is that removal of nkd has little effect if Wg has already been inactivated, because nkd is itself induced by the transduced Wg signal (Rousset et al., 2001; Zeng et al., 2000). The epistatic relationship between Axin and Wg in Drosophila has not yet been reported. However, embryos harboring a mutation in Drosophila APC (D-APC2) undergo expansion of en expression that is indistinguishable from that seen in nkd mutants (McCarty et al., 1999). The phenotype of a D-APC2 mutant embryo, similar to the nkd phenotype, requires intact Wg signaling: a D-APC2 wg mutant resembles a wg mutant (McCarty et al., 1999). Because nkd and D-APC2 have similar Wg-dependent phenotypes in Drosophila, vertebrate nkd genes may be acting in some tissues in an analogous fashion to vertebrate APC, a key tumor suppressor gene for colon cancer (Polakis, 1997).

We recently proposed that Drosophila Nkd might control the sensitivity of Wg-responsive cells by regulating the ability of Dsh to transmit these signals from the Fz receptors to the β-catenin “destruction complex” (Rousset et al., 2001). Our demonstration that the vertebrate Nkd proteins can interact with the three vertebrate Dsh-related proteins and, in the case of mNkd1, antagonize Wnt signals makes these nkd-related genes potentially pivotal regulators of Wnt signals in vertebrates. Future experiments will seek to understand the biochemical consequences of the Nkd/Dsh interaction during Wnt signaling, whether the nkd genes are inducible by vertebrate Wnt signals, and whether altering endogenous nkd levels in vertebrate animals results in abnormal development or disease attributable to Wnt hypersensitivity.

ACKNOWLEDGMENTS

We thank Lilijana Milenkovic, Janine Hartford, Matt Fish, and Mei Zhang for expert technical assistance; Chris Karovich and Rick Myers for radiation hybrid mapping of human nkd2; Ryan Rountree, Cathy Thut, David Kingsley, and Tony Oro for in situ hybridizations; Lara Collier, Bo Yu, and Roel Nusse for expression plasmids and cells; Bridg Hogan for the B5 d.p.c. mouse embryo library; Peter Klein, Tony Oro, Elizabeth Hick, and Clifford Tabin for helpful comments on the manuscript, and the rest of the Scott lab for continued advice and support. K.W. was supported by a HHMI Postdoctoral Fellowship for Physicians and by the NIH (K08 HD 01164-04); G.Z. was supported by HHMI; R.R. was supported by the Association pour la Recherche sur le Cancer and by the Human Frontier Science Program; M.P.S. is an Investigator of the Howard Hughes Medical Institute. HHMI supported this research.

REFERENCES


Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.


Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.


Received for publication February 22, 2001

Accepted February 23, 2001

Published online April 17, 2001