The protein kinases of *Caenorhabditis elegans*: A model for signal transduction in multicellular organisms

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***Caenorhabditis elegans* should soon be the first multicellular organism whose complete genomic sequence has been determined. This achievement provides a unique opportunity for a comprehensive assessment of the signal transduction molecules required for the existence of a multicellular animal. Although the worm *C. elegans* may not much resemble humans, the molecules that regulate signal transduction in these two organisms prove to be quite similar. We focus here on the content and diversity of protein kinases present in worms, together with an assessment of other classes of proteins that regulate protein phosphorylation. By systematic analysis of the 19,099 predicted *C. elegans* proteins, and thorough analysis of the finished and unfinished genomic sequences, we have identified 411 full length protein kinases and 21 partial kinase fragments. We also describe 82 additional proteins that are predicted to be structurally similar to conventional protein kinases even though they share minimal primary sequence identity. Finally, the richness of phosphorylation-dependent signaling pathways in worms is further supported with the identification of 185 protein phosphatases and 128 phosphoprotein-binding domains (SH2, PTB, STYX, SBF, 14-3-3, FHA, and WW) in the worm genome.**

Reversible protein phosphorylation plays a central role in regulating basic functions of all eukaryotes such as DNA replication, cell cycle control, gene transcription, protein translation, and energy metabolism. Protein phosphorylation is also required for more advanced functions in higher eukaryotes such as cell, organ, and limb differentiation, cell survival, synaptic transmission, cell–substratum and cell–cell communication, and to mediate complex interactions with the external environment. Because aberrant protein phosphorylation is commonly the cause of cancer and other human diseases, a comprehensive knowledge of the key enzymes that regulate these functions can provide the basis for novel therapeutic intervention strategies.

The genomic revolution promises to provide a new paradigm for drug discovery, allowing one to selectively target the molecular basis of human disease. The completion of the *Caenorhabditis elegans* genome sequence gives us an opportunity to decipher the molecular nature of its signal transduction machinery. Several global analyses of proteins and protein domains present in *C. elegans* have been presented elsewhere (1–4), revealing that protein kinases comprise the second largest family of protein domains in worms. The three most frequently occurring protein domains found in worms are seven transmembrane chemoreceptors (650 domains, 3.5% of genome), protein kinases (496 domains, 2.6% of genome), and zinc finger C4 domains, including nuclear hormone receptors (275 domains, 1.4% of genome). A more in-depth analysis has been performed on the 535 worm proteins containing zinc-binding domains, including the C4, C2H2, and C3HC4 ring finger types (3), and on the 83 worm homeobox transcription factors (4). Here, we present a comparative analysis of the enzymes and adaptor molecules that are the key components of the protein phosphorylation signaling network present in *C. elegans*.

Identification and Classification of *C. elegans* Protein Kinases. To identify worm protein kinases, we first used an HMMER 2.1.1 (http://hmmer.wustl.edu/) profile search against the 19,099 predicted worm proteins, the finished and unfinished *C. elegans* genomic sequence, and the worm chromosome assemblies. The nucleic acid databases were first translated in all six frames, and ORFs longer than 30 amino acids were parsed into a relational database. We generated a hidden Markov model based on 70 representative yeast and human protein kinases whose catalytic domains share <50% sequence identity with each other (5). Using a similar strategy, additional profiles were generated for other protein kinase-like domains (phosphoinositide kinases, atypical A6 kinases, diacylglycerol kinases, aminoglycoside resistance kinases, and microbial kinases), protein phosphatases, and domains capable of specifically binding to phosphotyrosine (P.Tyr) or phosphoserine/threonine residues (SH2, PTB, STYX, SBF, 14-3-3, FHA, and WW domains). Scripts were written for reassembly of contiguous exons identified from genomic sequence to generate the predicted catalytic domain sequence of each kinase. Pairwise BLAST 2.0 (ftp://ncbi.nlm.nih.gov/blast/executables/) analysis was performed to identify redundant entries, and putative protein kinases with low profile scores were manually inspected to determine whether they should be included in subsequent analyses.

This analysis generated a nonredundant list of 493 protein kinase-like proteins and 21 protein kinase gene fragments from worms. This number will continue to increase as the genome is completed and the final assembly of the six worm chromosomes is achieved. Of note, we found >40 kinase domains from genomic analysis that were absent in the 19,099 worm protein dataset. These omissions result from the limitations of current protein prediction algorithms. Furthermore, numerous entries had apparent internal deletions of conserved kinase motifs, likely attributable to inappropriately assigned splice junctions. These sequences were corrected before further classification. Many of the 19,099 proteins were alternate isoforms of the same gene, in which case we included

Abbreviations: PKA, protein kinase A; MAPK, mitogen-activated protein kinase; CDK, cyclin-dependent kinase; PTK, protein-tyrosine kinase; RTK, receptor protein-tyrosine kinases; CTK, cytoplasmic protein-tyrosine kinases; STAT, signal transducer and activator of transcription; IRS, insulin receptor substrate; NLK, NEMO-like kinase; APH, aminoglycoside phosphotransferases; PTP, protein-tyrosine phosphatase.

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REVIEW

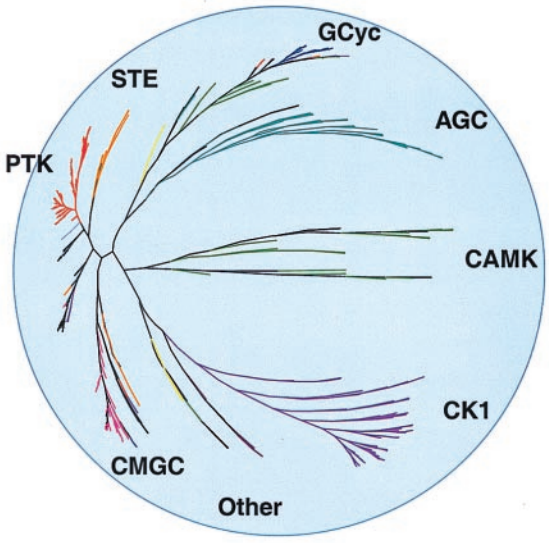

Fig. 1. Hyperbolic tree representation of *C. elegans* protein kinases. Major protein kinase groups are labeled in different colors. A JAVA tool for viewing this dendrogram can be found at www.kinase.com.

only one of the proteins in our final assessment. In determining the total number of protein kinases, the three proteins determined to contain dual catalytic domains were only counted once. Many of the protein ORFs truncated the extremities of the kinase domain proteins, frequently because of their location near the end of a cosmid clone. In these cases, we searched for N- or C-terminal domains on adjacent cosmids to assist in the subsequent classification. One challenge of genomic data mining is the presence of sequence repeats. Tandem repeats and inverted repeats account for 2.7 and 3.6% of the worm genome, respectively. In addition, worms contain large regions of tandem gene duplication, ranging from hundreds of bases to >100,000 bases (1). In some cases, the genes encoded within these regions are duplicated and have nearly identical sequences. Therefore, until the chromosome sequences are fully assembled, data-mining approaches may exclude some of these duplicated genes.

A multiple sequence alignment was generated from the predicted catalytic domains of 398 of these protein kinase, which share >15% amino acid identity with other entries. The aligned proteins were then clustered by using parsimony analysis, and the results were displayed as rooted and unrooted cluster dendrograms, and as kinase "retinograms" or hyperbolic trees using a JAVA display tool (Fig. 1 and www.kinase.com). The protein kinases were then classified into several kinase groups and families, based on relatedness within the kinase catalytic domain to other worm, yeast, and vertebrate protein kinases. Further classification was performed by searching for noncatalytic domains linked to the kinase domain, including predicted transmembrane regions, SH2 domains and SH3 domains, and Ig and fibronectin Type III domains.

Table 1 presents a summary of our classification of the 411 protein kinases and 82 protein kinase-like motifs. A more detailed table of these proteins, along with basic informatics tools for retrieval and alignment of these sequences can be found on our web site at www.kinase.com. Table 1 also summarizes the results of a similar analysis of the completed yeast genome and of an ongoing effort from publicly available human expressed sequence tag and genomic databases. From this classification, we can now determine which protein kinases are conserved between yeast and worms, we can speculate on the origin of the protein kinase superfamily, and we can identify kinases that are yeast-specific and those that are restricted to higher eukaryotes. We tentatively identify "worm-specific" protein kinases, based on their absence from current

Table 1. Summary and classification of phosphoprotein signaling molecules in worms, budding yeast, and humans

Superfamily	Group	Worm	Fragments worm	Yeast	Human
Protein kinase	AGC	30	1	17	100
	CAMK	32	0	21	83
	CKI	87	7	4	5
	CMGC	42	0	24	62
	Other	62	6	29	163
	STE	28	0	15	63
	PTK	92	5	0	100
	"Worm"	27	2	0	0
	"Yeast"	0	0	4	0
	"Microbial"	7	0	6	5
	Atypical	4	0	4	11
	All	**411**	**21**	**124**	**592**
PK-like	Gcyc	26	0	1	8
	PIK	12	0	10	20
	DAGK	7	0	2	8
	YLK1	30	0	0	0
	Choline K	7	0	2	2
	All	**82**	**0**	**15**	**38**
Phosphatase	cPTP	57	4	3	25
	RPTP	26	14	0	22
	DSP	26	0	16	51
	STP	65	0	18	21
	IPP	11	0	7	7
	All	**185**	**18**	**44**	**126**
PP-Binding	SH2	73	1	1	>137
	PTB	16	0	0	>47
	STYX (DSP)	1	0	5	2
	SBF (MTM)	2	0	0	3
	14-3-3	3	0	2	>6
	FHA	11	0	14	>20
	WW	22	0	5	>32
	All	**128**	**1**	**27**	**>247**
Other	Cyclins	34	0	23	>21

mammalian expressed sequence tag and nucleic acid databases. However, a final assessment will have to await completion of the *Drosophila* and human genome sequences. We also elaborate on some of the protein kinases and signaling pathways that evolutionarily appear only in more complex organisms such as vertebrates.

In this review, we use the term "orthologues" to refer to proteins of different species that are believed to have a common ancestor and have an evolutionarily conserved function. Orthologous proteins typically have similar domain structure and share extended sequence similarity outside of their catalytic domains. Homologous proteins also share extended sequence similarity, but to a lesser degree than orthologues, and are not expected to complement one another functionally. However, within large protein superfamilies such as protein kinases, G protein coupled receptors, and nuclear hormone receptors, there is not a single expectation value that can be used to categorize all members definitively, and final classification will require experimental validation.

Yeast- and Fungal-Specific Kinases. The first complete eukaryote sequence, that of the budding yeast *Saccharomyces cerevisiae*, was reported in 1996 (6). Shortly thereafter, we presented a comprehensive analysis and classification of yeast protein kinases (7). Now, with the availability of a second eukaryotic genome, *C. elegans*, we can perform a similar analysis and make more informed general-

izations on which of these protein kinases are unique to yeast or fungi, and also on which protein kinases evolved during the emergence of multicellular organisms and are therefore not represented in yeast or fungi.

We now identify a total of 24 yeast-specific protein kinases and an additional 3 that are currently restricted to yeast and worms. Originally we defined four protein kinase subfamilies, containing a total of 18 members, to be yeast specific [protein kinase A (PKA)-related, RAN, ELM, and NPR/HAL5 families]. These remain yeast- or fungal-specific, as no close homologues are present in worms, and none have yet been described in vertebrates. However, the ELM family could be considered as a subfamily of the CAMK group. Rim15 is a yeast-specific kinase that is related to *Schizosaccharomyces pombe* Cek1, and its similarity to budding yeast YNL161w places it as a distant member of the NDR family kinases. Two other protein kinase subfamilies, containing a total of five members, were originally recognized as having only distant homologues in higher organisms (NEK-like and PIM-like families). The prototype of the NEK-like family, YNL020C, has a homologue in worms, but not in mammals, although its C-terminal tail has a predicted coiled-coil structure related to numerous mammalian protein kinases (e.g., SLK/PLKK, TAK1). The two yeast PIM-like family members have catalytic domains related to worm and mammalian protein kinases, but have a unique N-terminal domain.

Members of the NPR/HAL5 family are involved in ion homeostasis, polyamine transport, nutrient uptake, and response to nitrogen starvation, whereas Elm1 initiates a protein kinase cascade controlling pseudohyphal growth (8). Members of the RAN family are related to fission yeast Ran1/Pat1, which regulates the switch between vegetative growth and meiosis. Because these are fungal-specific responses, it is not surprising that these protein kinases are restricted to lower eukaryotes.

A second set of "unique" yeast protein kinases was originally defined because they had no close homologues in other species (7). Most of these yeast protein kinases now have both worm and vertebrate orthologues (Cdc5, Ipl1, Ire1, Vps15, YGL180W/Apg1, Swe1, Spk1, Gcn2, YBR274W, YGR262C, and Bub1). Exceptions among this list of unique yeast protein kinases are YPL236C and Mps1, which have orthologues in humans, but not in worms; YKL116C, which is distantly related to the EMK-family, yet has only weak homologues in worms and humans; and YKL171W, YGR052W, and YPR106W, which remain yeast specific protein kinases. Two sequences that were excluded from our previous analysis of yeast protein kinases deserve mention. The budding yeast protein Iks1 can be classified as a yeast-specific protein kinase because it still has no homologues in worms or other species whereas another yeast kinase-like sequence, SCY1, has orthologues in *C. elegans* and *Arabidopsis*, but none thus far in vertebrates. A *S. pombe* protein, which is distantly related to SCY1, also has a single worm orthologue.

Worm-Specific Protein Kinases. Which protein kinases are specific to worms? Protein kinases that are absent from yeast yet present in worms are likely to be involved in the complex signal transduction pathways that are required for the existence of multicellular organisms. These might include protein kinases involved in cell–substratum and cell–cell adhesion, transmembrane signaling in response to humoral factors, protein kinases involved in cell survival or programmed cell death, and protein kinases whose signals regulate metazoan-specific transcription factors, particularly those containing Zn-finger domains.

In the absence of complete genome sequences of other multicellular eukaryotes, we tentatively classify 165 protein kinases (plus 9 protein kinase fragments) as worm-specific. The majority (134, 80%) fall into three groups (CK1, FER, and KIN-15) whereas the others are distant members of common protein kinase families or belong to worm specific subfamilies. Five protein kinase subfamilies, containing a total of 12 members, can tentatively be defined as worm-specific (C04G2.10, K08B4.5, K09C6.7, R107.4, and ZK177.2-families). An additional 15 unique worm protein kinases are also identified, which to date have no close homologues in yeast, worms, or in higher organisms. However, mammalian homologues of some of these worm protein kinases are already beginning to appear in publicly available expressed sequence tag databases, and assignment of a protein kinase as being truly worm-specific will have to await the completion of the *Drosophila* and human genome sequences.

Members of four other protein kinase or kinase-like subfamilies are disproportionately represented in worms compared with humans. Clusters of 5–9 members of each of these families are localized to short regions (<1 megabase) of chromosomes II and IV, suggesting they may each have expanded as a result of extensive tandem gene duplication. The chromosomal density of protein kinases is graphically depicted on our web site at www.kinase.com. The four gene families are the CK1-family, the KIN-15-family of receptor protein-tyrosine kinases, the FER-family of cytoplasmic protein-tyrosine kinases, and the kinase-like domains of the receptor guanylyl cyclases.

CK1 family. The worm genome contains 87 CK1 (casein kinase I) members (plus 7 additional partial catalytic domains) whereas there are only 4 known members in budding yeast and 6 in humans. Genetic evidence from the yeast homologues suggests CK1s may be involved in DNA repair and cell division, and mammalian CK1s have been shown to phosphorylate p53 in G1 and G2, possibly affecting cell sensitivity to DNA damage at these checkpoints (9). Little is known regarding the function of CK1s in worms, but the enormous arborization and diversification of this kinase family may be an adaptation allowing for enhanced DNA repair in response to excessive exposure to environmental mutagens.

KIN-15/16 family. C. elegans contains 16 members of a unique family of receptor protein-tyrosine kinases whose presence to date is restricted to this species. These transmembrane proteins have unusually short (<50-aa) extracellular domains, and many are clustered within the genome, as though they arose through tandem gene duplication. The prototype members of this family, KIN-15 and KIN-16, are expressed in the hypodermal syncytium, which expands by cell fusion during larval development (10). Compared with wild-type worms, KIN-15 and KIN-16 deletion mutants produce fewer embryos and rarely develop into adults, but, when they do mature, they typically exhibit extrusion of the gonads through the vulva (11). Therefore, KIN-15/16 appear to be essential genes, yet may undergo variable compensation by 1 of the 14 other homologues. One of the KIN-15 clusters is interspersed with chitinase genes, which are known to function in cell wall morphogenesis during the molting process and in fungal resistance. Expansion of this region may have been necessary during evolution to facilitate this aspect of larval development. An alternative function for KIN-15-family kinases is suggested by the fact that overexpression TKR-1 (C08H9.5) causes a 40–100% extension of life expectancy in worms (12). Unlike other life extension (*age*) mutants, TKR-1 transgenics do not form dauers, and their longevity has been attributed to an increased resistance to ultraviolet and thermal stress.

FER family. The worm genome contains 42 members (plus 2 additional partial catalytic domains) of the FER-family of single SH2-containing cytoplasmic protein-tyrosine kinases. Most of these genes are interspersed throughout the worm genome; however, nine members reside within a 1.1-megabase region on chromosome IV. Unfortunately, no literature is available on the function of any of these protein kinases in worms, but the two mammalian homologues, FER and FES, have been demonstrated to play a role in cell adhesion, to signal downstream of cytokine receptors, and to function as oncogenes (13). Conceivably, additional human representatives will be revealed on completion of the human genome sequence, possibly with restricted expression. Alternatively, their function may be replaced in humans by expansion and

diversification of non-FER cytoplasmic protein-tyrosine kinases, of which worms have only 10 whereas humans have at least 34. Most evident is a dramatic expansion of SRC-family kinases and emergence of ZAP70 and JAK family kinases in higher eukaryotes that are not found in the worm genome.

Conserved Metazoan Protein Kinase Signaling Transduction Pathways. Worms provide an elegant model system for studying signal transduction. This transparent animal is comprised of 959 somatic cells plus 131 cells destined for programmed cell death. The *C. elegans* hermaphrodite contains 302 neurons and 81 muscle cells and has a brain, reproductive system, and digestive tract (ref. 14; http://dauerdigs.biosci.missouri.edu/Dauer-World/Wormintro.html). It provides a complex yet tractable system for studying development, metabolism, aging, and behavioral responses to a number of stimuli. Regulation of many of these processes is carried out through signal transduction pathways that are also present in humans. Not surprisingly, all of the major protein kinase groups found in worms are also conserved in humans (15). The number of protein kinases classified into each major group from yeast and worms, along with a current estimate from humans, is provided in Table 1. These numbers represent a current analysis, but new protein kinases are being discovered every month as the worm genome sequencing project continues. Some of these entries may also represent pseudogenes containing frameshifts that result in incomplete translation into a full kinase catalytic domain.

AGC Group. The AGC group of worm protein kinases contains representatives of many of the known types of cyclic nucleotide-dependent, NDR or DBF2, and ribosomal S6 kinase families. Worms also contain members of the cGMP-dependent kinase (PKG), RSK, and G-protein coupled receptor kinase families that are absent from budding yeast. Two of the S6 kinase members have dual catalytic domains similar to vertebrate RSK enzymes, where the N-terminal domain clusters into the AGC group and the C-terminal kinase domain is most related to the CaMK group. Worms have four members of the AKT family, two being close orthologues of mammalian AKT1/PKB/RACα, and two related to the AKT upstream kinase, PDK1. AKT is a mammalian protooncoprotein regulated by phosphatidylinositol 3-kinase (PI3-K), which appears to function as a cell survival signal to protect cells from apoptosis (16). Insulin receptor, RAS, PI3-K, and PDK1 all act as upstream activators of AKT whereas the lipid phosphatase PTEN functions as a negative regulator of the PI3-K/AKT pathway (17). Downstream targets for AKT-mediated cell survival include the proapoptotic factors BAD and Caspase9 and transcription factors in the forkhead family, such as DAF-16 in the worm. AKT is also an essential mediator in insulin signaling, in part because of its use of GSK-3 as another downstream target. Each of these components of the AKT/PI3-K pathway is conserved in worms, providing a powerful system for genetic dissection of a major cell survival signal.

The cAMP-dependent protein kinases (PKA) consist of heterotetramers comprised of two catalytic (C) and two regulatory (R) subunits, in which the R subunits bind to the second messenger cAMP, leading to dissociation of the active C subunits from the complex. Worms have two PKA catalytic domains and two regulatory subunit genes (R07E4.6 and ZK370.4). Additional cNMP-binding domains are present in the two worm representatives of the PKG family, in several cNMP-gated ion channels, and in a cAMP-regulated guanine nucleotide exchange factor (T20G5.5).

CaMK Group. In the CaMK group, the most abundant representatives include Ca^{2+}/calmodulin-regulated and AMP-dependent protein kinases and EMK-related kinases. Worms also contain members of the death-associated protein kinase, mitogen-activated protein kinase (MAPK)-associated protein kinase, myosin light chain kinase, and phosphorylase kinase families that are absent from budding yeast. All of these protein kinase families have likely evolved as a result of the demands of multicellularity and the emergence of complex organ systems. For example, even though yeast have myosin homologues, they lack myosin light chain kinases. These protein kinases have presumably evolved to regulate myosin during muscle contraction. A worm contig still under construction appears to contain a phosphorylase kinase catalytic γ subunit orthologue, consistent with the presence of two orthologues of the noncatalytic phosphorylase kinase α subunits, which facilitate calmodulin-binding and are required for activation of the mammalian holoenzyme.

Worms lack a homologue of the mammalian Trio-family kinases. Trio is a large multidomain protein kinase containing Ras and Rho guanine exchange factor domains in addition to PH, SH3, and spectrin domains (18). Trio may link Rho and Rac signaling pathways and appears to be involved in the cytoskeletal changes required for cell migration. Although worms lack a member of this kinase family, they do have at least two proteins related to the entire noncatalytic domain of Trio (UNC-73 and F55C7.7).

We have also identified a forkhead homology (FHA) domain-containing CHK2 orthologue in worms. In yeast, Spk1/Rad3 functions as a DNA damage checkpoint sensor through its FHA domain interacting with phosphorylated Rad9 (19). Although no close orthologue of Spk1 exists in metazoans, this function appears to be replaced by CHK2/CDS1, which is phosphorylated in response to DNA damage and may work in conjunction with CHK1 kinase to phosphorylate CDC25C to prevent premature entry into mitosis (20).

CMGC Group. In the CMGC group of serine/threonine kinases, all of the main subfamilies are conserved between yeast, worms, and mammals, including cyclin-dependent kinase (CDK), MAPK, GSK-3, and CLK. An exception is the RCK family, which is absent from yeast but has two members in worms and at least seven in humans. The worm RCK kinases are most similar to mammalian MAK, or male germ cell-associated kinase, which has been implicated in spermatogenic meiosis and in signal transduction pathways for sight and smell. Worms have 14 CDKs (compared with 5 CDKs in yeast) including orthologues of CDC2, CDK3, CDK5, CDK7, and CDK8, and contain 34 cyclins, compared with 23 in budding yeast (Table 1), including one cyclin H orthologue, which we predict will interact with worm CDK-7 to generate a functional cyclin-activated kinase.

Worms have 14 MAPKs, compared with 6 in yeast and at least 14 in humans. The worm MAPKs include representatives of each of the major types of MAPKs: ERK/MAPK, JNK/SAPK1, p38/SAPK2, BMK/ERK5, and NEMO-like kinase (NLK) (21). In budding yeast, three protein kinase families (the prototypes being Ste20, Ste11, and Ste7) function upstream of the MAPKs to generate at least four distinct MAPK signaling pathways that mediate the response to pheromone, nutritional starvation, and cellular or osmotic stress. In multicellular organisms, these MAPK cascades have evolved to mediate responses to diverse signals including growth factors, mitogens, hormones, and cytokines, in addition to the more primitive stress responses to anoxia, heat shock, and osmotic stress.

STE Group: MAPK Pathways. The STE family refers to the three classes of protein kinases that lie sequentially upstream of the MAPKs. In worms, this group includes 10 STE7 (MEK or MAPKK) kinases, 2 STE11 (MEKK or MAPKKK) kinases, and 12 STE20 (MEKKK) kinases. Based on the number of MAPK and STE-family kinases in *C. elegans*, we predict worms will contain at least 8–10 MAPK pathways. In humans, several protein kinase families that bear only distant homology with the STE11 family also operate at the level of MAPKKKs, including RAF, MLK, TAK1, and COT. Except for COT, worms also have orthologues of each of these kinases. Because crosstalk takes place between protein

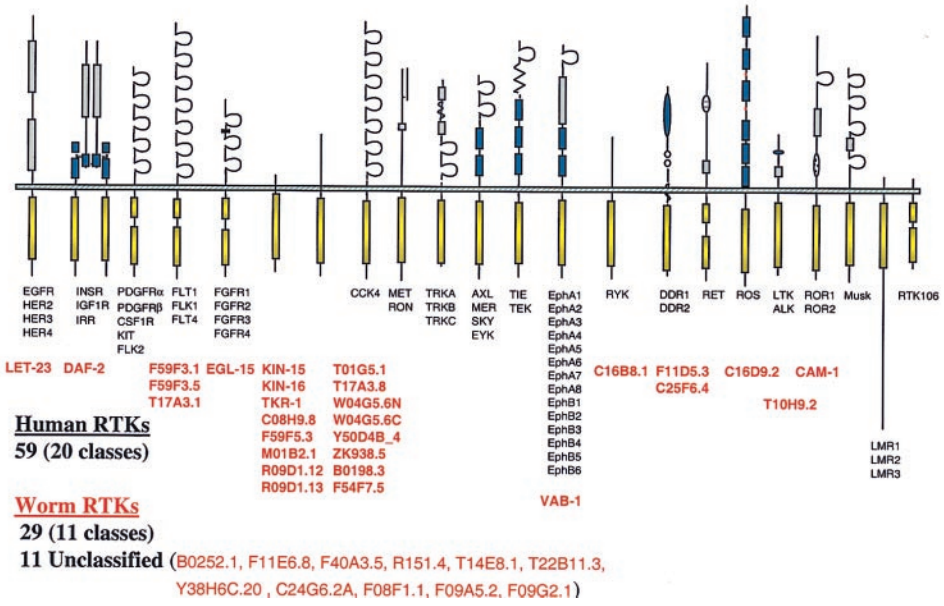

Fig. 2. Schematic representation of the human and *C. elegans* receptor protein-tyrosine kinase families. Catalytic domains are shown in yellow. The names of the human RTKs are in black, and the names of the worm RTKs are in red.

kinases functioning at different levels of the MAPK cascade, the large number of STE family kinases could translate into an enormous potential for upstream signal specificity and diversity.

Protein-Tyrosine Kinase Group: Receptor Protein-Tyrosine Kinases (RTKs). The largest group of protein kinases in worms are the protein-tyrosine kinases (PTKs), with 92 members and 5 fragments. We predict this will also remain the largest group of protein kinases in higher eukaryotes, including humans, where the current count is ≈100. These numbers are impressive when one considers that this family is absent from budding yeast. Yeast, however, do have a "budding" tyrosine phosphorylation signaling system, with several dual-specificity kinases (CLK-like, Ste7/MEK family, Swe1, Spk1/Rad53, Mps1), an atypical A6 PTK, 3 protein-tyrosine phosphatases, 16 dual-specificity and low molecular weight phosphatases, and 6 "infant" P.Tyr-binding proteins comprising an apparently nonfunctional SH2 domain protein and 5 phosphatase-like STYX domains. Budding yeast lack PTB domains, and none of the six potential P.Tyr-binding domains have been functionally verified.

The 92 worm PTKs can be further classified into receptor protein-tyrosine kinases (RTKs) and cytoplasmic protein-tyrosine kinases (CTKs) based on the presence or absence of a transmembrane domain and SH2 or SH3 domains. Based on this analysis, the worm genome contains 40 RTKs and 52 CTKs. The 40 RTKs include 16 members of the worm-specific KIN-15-family, 13 RTKs with orthologues representing 10 of the 20 families of human RTKs, and 11 RTKs that remain unclassified with no identifiable mammalian counterpart (Fig. 2). Genetic studies in worms support the classification of five of these worm–human pairs, including LET-23/EGF receptor, DAF-2/insulin receptor, EGL-15/FGF receptor, CAM-1/ROR1 receptor, and VAB-1/EPH receptor, and each of these orthologous pairs mediates similar functions in worms and man, with specificity for epidermal, metabolic, mesodermal, and neuronal signaling pathways.

Based on extracellular domain homologies, we also predict three worm orthologues of PDGFR/FLK/VEGFR, two for DDR, and one each of RYK, ROS, and LTK/ALK. Two of the unclassified RTKs have weak similarity to MET, but not enough to warrant inclusion into this family. Missing in *C. elegans* are TRK/nerve growth factor receptors, AXL/TYRO3, TIE/angiopoietin receptor, RET/GDNF receptor, and MUSK family members. Identification of three members of the PDGFR/VEGFR family is significant, as they emerged only through analysis of the genomic data and failed to be properly identified from a recent analysis of the predicted 19,099 proteins. Each of these receptors contains multiple Ig-like extracellular domains and a single split kinase domain with closest homology to human FLT1/VEGFR1 and the *C. elegans* KIN-15 family. However, they are likely to represent early ancestors to both the FLK and PDGFR kinase lineages. Expression of the mammalian FLK/VEGFR RTKs is primarily restricted to endothelial cells, and they play important roles in the early differentiation of hematopoietic and endothelial lineages as well as in normal and pathologic angiogenesis in the adult. However, because worms lack a vasculature, the function of these receptors is not obvious. The formation of mammalian vasculature is reminiscent of the process by which networks of branching tubes develop into the lung and kidneys. Invertebrate VEGFRs may therefore be involved in processes that later evolved into a program for limb and organ development in vertebrates.

Surprisingly little is known about how the ligand-activated VEGFRs mediate these effects. Gene knockout studies in mice suggest that A-RAF or MEKK1 may function downstream of VEGFRs, and recent evidence implicates the involvement of STATs (signal transducer and activator of transcription) in VEGFR signaling (22). Genomic analysis reveals two worm orthologues of STATs (Y51H4, Y43D4 unfinished and F58E6.1), making the VEGFR-STAT association an attractive area for further investigation. STATs contain an SH2 domain, a tyrosine phosphorylation domain, and a DNA-binding domain, and function in a unique JAK-STAT signaling pathway. Extensive studies in mammalian systems have established a model in which JAK kinases are constitutively bound to the cytoplasmic portion of cytokine receptors and are activated on receptor dimerization, facilitating recruitment of STATs to the receptor complex. Subsequent STAT phosphorylation leads to their dimerization and translocation to the nucleus, where they function directly as transcription factors. *Drosophila* and *Dictyostelium* STATs both regulate cell division and pattern formation (23, 24). *Drosophila* STAT has been genetically and biochemically linked to a JAK-STAT signal transduction pathway that regulates pair-rule genes and hematopoiesis. *Dictyo-*

stelium STAT plays an essential role during the differentiation and aggregation of independent spore cells into stalk cells in response to the chemical signal referred to as differentiation-inducing factor. Furthermore, the *Dictyostelium* AX2 PTK has a second kinase-like domain found only in JAK-family kinases, suggesting the existence of a signaling network similar to that in flies and mammals. However, worms have no cytokines, no cytokine receptors, and no JAK-family kinases. Possibly, the JAK kinase function is replaced by a worm-specific FER kinase, or the STATs may have initially evolved to serve an alternative purpose. Mammalian STATs are also involved in signaling through receptors for growth factors such as EGF, PDGF, VEGF, and angiopoietins. Because the EGF and VEGF signaling systems are present in worms, it is tempting to speculate that these represent the primordial raison d'être for STATs.

In general, related RTKs bind related ligands. In humans, there are at least 12 ligands, encoded by 10 genes, that have been shown to bind selectively to at least one of the four known EGFR-family members. Each of these ligands shares a conserved six-cysteine pattern in its receptor binding domain. In worms, LIN-3 has been shown to function as a LET-23 ligand. Although EGF motifs are prevalent in worms, we have identified three EGF-like proteins (F58G4.4, Y69H2.2, YG70G10A.2) that, in addition to the six cysteines, conserve many of the crucial receptor-binding residues and are juxtaposed next to a putative transmembrane domain, in a pattern similar to all known EGFR-family ligands. Worms also contain at least 3 FGF-like ligands, 12 insulin-like ligands (many more on inclusion of relaxin-related ligands), 2 distant homologues of VEGF, and 4 ephrin-related ligands, some of which would be predicted to bind to their cognate receptors.

Orthologues of other RTK ligands prove more difficult to identify empirically. We see no evidence for a bona fide PDGF or NGF, and searches for ligands for MET, TIE, and AXL-family RTKs are confounded by their similarity to plasminogen, fibrinogen, and fibrillin, respectively. Furthermore, except for weak homologues of MET, these three RTK families are absent from worms. Nevertheless, the significance of a putative Ang2-like protein (Y43C5A.2) in the absence of a TIE-family RTK remains to be determined.

Protein-Tyrosine Kinase Group: CTKs. Most of the 52 CTKs in worms belong to the single SH2-containing FER family. Of the remaining 10 CTKs, there are 2 orthologues of the SH3-containing ACK, and 1 each of FYN (SRC family CTK), FRK, CSK, ABL, and FAK, plus 3 unclassified CTKs. In vertebrates, CSK negatively regulates FYN-family kinases by phosphorylation of a C-terminal tyrosine facilitating a conformational change through an intramolecular SH2-P.Tyr interaction (25). We predict a similar functional interaction between worm FYN and CSK. Co-evolution of this regulatory pair suggests even early metazoans required a means to dampen signaling through CTKs. Notably absent in worms are protein kinases related to the ZAP70 and JAK CTKs, whose primary role in mammals is in signaling through the T cell and cytokine receptors, both of which represent more specialized pathways not present in worms. Humans have eight SRC-family kinases whereas worms have only one. This redundancy has confounded efforts to dissect out the precise role of these CTKs in human biology, often requiring "triple knockouts" to demonstrate a deficiency. The simplicity of non-FER-like CTKs in worms may be helpful in placing these CTKs within specific signaling cascades.

Protein-Tyrosine Kinases: Adaptor and Docking Molecules. Ligand activation of RTKs results in tyrosine phosphorylation of both the receptor itself (autophosphorylation) and of downstream substrates. These phosphorylated tyrosines then function as attachment sites for proteins containing SH2 and other P.Tyr-binding domains. We have identified 74 proteins containing a total of 77 SH2 domains in worms. The majority of these SH2 domains are in CTKs, two are present in a SHP2-related PTP, and the remainder are predicted to represent orthologues of a variety of adaptor molecules, including phospholipase Cγ, CBL, CIS4/SOCS5, CRK, NCK, SEM-5/GRB2, SHC, tensin, STAT, and VAV. Worms also contain at least 16 PTB domains, which in some cases have been found to interact specifically with tyrosine phosphorylated proteins. Worm PTB-containing proteins include orthologues of SHC, which also contains an SH2 domain, neuronal transmembrane protein X11, and an insulin receptor substrate (IRS) family member. The mammalian X11 PTB domain does not to bind to P.Tyr, so we anticipate only a few of these worm domains will function as P.Tyr-binding domains. Additional potential phosphoprotein-binding domains identified in worms include three 14-3-3 domains, 22 WW domains, and 11 FHA domains.

IRS-1 and IRS-2 are major substrates of the insulin receptor RTK in mammals, and disruption of IRS-2 in mice leads to metabolic defects similar to diabetes. Worms have multiple insulin-like peptides, a receptor, and an IRS orthologue, demonstrating the early origins of metabolic regulation in multicellular organisms. The presence of such a diverse array of adaptor molecules underscores the utility of worms as a model for understanding mammalian signal transduction.

Other Protein Kinases. Approximately 15% of the worm protein kinases do not fall into one of the six major groups but include smaller families with representatives in higher eukaryotes, including CHK1, DYRK, MLK, TAK1, PIM, RAF, STKR, and the mitotic kinases (BUB1, AURORA, PLK, and NIMA/NEK). Recent genetic and biochemical data place TAK1 (transforming growth factor β-associated kinase) on a MAPK-like pathway at the level of a MAPKKK acting upstream of the MAPK-family member NLK. The worm orthologues of TAK1 and NLK regulate Wnt-mediated cell polarization during embryogenesis (21). Biochemical data also demonstrate that this MAPK-like pathway negatively regulates Wnt signaling because NLK phosphorylates the TCF/LEF HMG transcription factors, thereby inhibiting Wnt-regulated binding of the β-catenin-TCF complex to DNA. Both of these pathways are conserved between mammals and worms. The likely orthologous human/worm pairs on the TAK1 MAPK-like pathway include TAK1/MOM-4, NLK/LIT-1, and TCF4/POP-1. Upstream regulators may include TGFβ1/DBL-1, TGFβ type I receptor/SMA-6, TGFβ type II receptor/DAF-4 (worms have three receptor serine kinases). Additional components of the Wnt-signaling pathway, such as cadherin, the adenomatous polyposis coli tumor suppressor gene (APC), disheveled, and GSK-3 kinase are also present in worms, suggesting that there may be a primordial connection between polarized control of cell division/migration and cellular transformation in vertebrates (26).

Microbial-Like Kinases: Origin of Protein Kinases? The availability of the sequence of the first complete metazoan genome, combined with the sequence of budding yeast and several prokaryotic and *Archaea* genomes, provides an excellent opportunity to reassess current theories on the evolutionary origin of protein kinases. Pkn1 is a bacterial protein kinase-like sequence first described in the Gram-negative bacteria *Myxococcus xanthus*, which functions in growth and differentiation and in the ability of this prokaryote to form a fruiting body in response to nutrient starvation. Pkn-related proteins are present in other prokaryotes, including *Streptomyces*, *Bacillus*, *Mycobacterium*, *Pseudomonas*, *Chlamydia*, and *Synechocystis*, where they are involved in virulence, secondary metabolism, sporulation, and complex growth cycles (27). However, there are no Pkn homologues in bacteria with less complex life cycles, such as *Escherichia coli*, and *Haemophilus influenzae*, or in any *Archaea*, suggesting they may have been acquired by horizontal transmission from an early eukaryote, and are unlikely to represent the ancestral founders to protein kinases.

In our kinase profile searches of the worm genome, we detected several entries with low profile scores, yet with significant (E value $< 10^{-2}$) random expectation (E) values. Most of these contained similarity to kinase subdomains I, II, and VI, containing

the consensus GxGxxGxV, VAVK, and HxDxxxxN motifs, respectively. Upon further analysis, many of these entries could be classified into distinct families designated ABC1, RI01, YGR262, diacylglycerol kinase, choline/ethanolamine kinases, and the YLK1-antibiotic resistance kinases. The first three families are named after their prototypic members in *S. cerevisiae* (27).

Worms contain three proteins related to the budding yeast ABC1. The yeast protein is required for the assembly of the mitochondrial cytochrome *c* reductase complex, which functions as an electron carrier during oxidative phosphorylation to generate ATP (28). ABC1 homologues are present in numerous prokaryotes, including *Mycobacterium*, *Clostridium*, *Rickettsia*, *Synechocystis*, *Azobacter*, and *Enterobacteriaceae* such as *E. coli* and *Providencia stuartii*, in addition to the *Archaea*, *Methanobacterium*. ABC1-like proteins are also present in eukaryotes, including fission yeast, *Arabidopsis*, worms, and mammals. Although ABC1 homologues are absent from bacteria such as *Mycoplasma*, *Bacillus*, *Haemophilus*, *Helicobacter*, and spirochetes, their presence in other prokaryotes, *Archaea,* and eukaryotes positions them as likely representatives of the primordial protein kinase, which was the progenitor of the eukaryotic protein kinase family. Based on their recognized role in mitochondrial ATP production and because they maintain many of the structurally important residues and motifs involved in ATP binding, the ABC1-family proteins may either bind ATP or act as phosphotransferases. Conceivably, the ABC1 proteins transfer phosphate to proteins as part of a feedback loop to sense mitochondrial ATP levels.

The RI01 family has three representatives in worms and is named after one of the two homologues in budding yeast. There are also representatives from several *Archaea* species, but none from bacteria, making them a less attractive candidate as a progenitor to the protein kinase lineage.

Atypical Protein Kinases and Protein Kinase-Like Domains. Worms contain 26 kinase-like domains present in receptor guanylyl cyclases (there are 10 additional soluble guanylyl cyclases), and at least 7 diacylglycerol kinases, 7 choline/ethanolamine kinases, and 30 YLK1-related antibiotic resistance kinases. Each of these families contain short motifs that were recognized by our profile searches with low scoring E-values, but *a priori* would not be expected to function as protein kinases. Instead, the similarity could simply reflect the modular nature of protein evolution and the primal role of ATP binding in diverse phosphotransfer enzymes. However, two recent papers on a bacterial homologue of the YLK1 family suggests that the aminoglycoside phosphotransferases (APHs) are structurally and functionally related to protein kinases (28, 29). There are over 40 APHs identified from bacteria that are resistant to aminoglycosides such as kanamycin, gentamycin, or amikacin. The crystal structure of one well characterized APH reveals that it shares >40% structural identity with the two-lobed structure of the catalytic domain of cAMP-dependent protein kinase (PKA), including an N-terminal lobe composed of a five-stranded antiparallel β sheet and the core of the C-terminal lobe, including several invariant segments found in all protein kinases (29). APHs lack the GxGxxG normally present in the loop between β strands 1 and 2 but contain 7 of the 12 strictly conserved residues present in most protein kinases, including the HGDxxxN signature sequence in kinase subdomain VIB (29). Furthermore, Daigle *et al.* (30) have demonstrated that this APH also exhibits protein-serine/threonine kinase activity, suggesting that the worm YLK1-related molecules may indeed be functional protein kinases.

The eukaryotic lipid kinases (PI3Ks, PI4Ks, and PIPKs) also contain several short motifs similar to protein kinases but otherwise share minimal primary sequence similarity. However, once again, structural analysis of PIPKIIβ defines a conserved ATP-binding core that is strikingly similar to conventional protein kinases (31). Three residues are conserved among all of these enzymes, including (relative to the PKA sequence) Lys-72, which binds the α-phosphate of ATP, Asp-166, which is part of the HRDLK motif, and Asp-184, from the conserved Mg^{2+} or Mn^{2+} binding DFG motif (31). The worm genome contains 12 phosphatidylinositol kinases, including 3 PI3-kinases, 2 PI4-kinases, 3 PIP5-kinases, and 4 PI3-kinase-related kinases. The latter group has four mammalian members (DNA-PK, FRAP/TOR, ATM, and ATR), which have been shown to participate in the maintenance of genomic integrity in response to DNA damage and exhibit true protein kinase activity, raising the possibility that other PI-kinases may also act as protein kinases. Regardless of whether they have true protein kinase activity, PI3-kinases are tightly linked to protein kinase signaling, as evidenced by their involvement downstream of many growth factor receptors and as upstream activators of the cell survival response mediated by the AKT protein kinase.

There are several proteins with protein kinase activity that appear structurally unrelated to the eukaryotic protein kinases. These include *Dictyostelium* myosin heavy chain kinase A, *Physarum polycephalum* actin-fragmin kinase, the human A6 PTK, human BCR, mitochondrial pyruvate dehydrogenase and branched chain fatty acid dehydrogenase kinase, and the prokaryotic "histidine" protein kinase family. Worms lack representatives of the actin-fragmin kinases, BCR, and bacterial histidine kinases yet do contain a single representative of the other classes of atypical kinases and two homologues of the A6-related PTKs. The single worm orthologue of the *Dictyostelium* myosin heavy chain kinase A (32) proves to be the worm eukaryotic elongation factor 2 kinase (33). The slime mold, worm, and human eukaryotic elongation factor 2 kinase homologues have all been demonstrated to have protein kinase activity, yet they bear little resemblance to conventional protein kinases except for the presence of a putative GxGxxG ATP-binding motif (33).

The so-called histidine kinases are abundant in prokaryotes, with >20 representatives in *E. coli*, and have also been identified in yeast, molds, and plants. In response to external stimuli, these kinases act as part of two-component systems to regulate DNA replication, cell division, and differentiation through phosphorylation of an aspartate in the target protein (34). To date, no "histidine" kinases have been identified in metazoans, although mitochondrial pyruvate dehydrogenase (PDK) and branched chain α-ketoacid dehydrogenase kinase are related in sequence. PDK and branched chain α-ketoacid dehydrogenase kinase represent a unique family of atypical protein kinases involved in regulation of glycolysis, the citric acid cycle, and protein synthesis during protein malnutrition. Structurally, they conserve only the C-terminal portion of "histidine" kinases, including the G box regions. Branched chain α-ketoacid dehydrogenase kinase phosphorylates the E1α subunit of the branched chain α-ketoacid dehydrogenase complex on Ser-293, proving it to be a functional protein kinase (35). Although no bona fide "histidine" kinase has yet been identified in worms or humans, they do contain PDK homologues (one in worms and five in humans). However, the paucity of PDKs in worms makes it unlikely that they fill in for the absence of "histidine" kinases in metazoans. Instead, these signaling cascades have more likely been replaced by pathways initiated through RTKs.

Based on these examples of atypical protein kinases present in the worm genome, we predict additional worm protein kinases will be functionally identified that lack any of the obvious motifs conserved in the conventional members. Indeed, various biochemical data point to the existence of true histidine, lysine, and arginine kinases in metazoans, yet their structural identity remains a mystery.

Protein Phosphatases. Because of their important role in signal transduction, it is not surprising that the activity of protein kinases must be tightly regulated. This is accomplished through autoinhibition, autophosphorylation, transphosphorylation, dimerization, and cellular localization. Equally important is the role of protein phosphatases, which act to remove these regulatory phosphates from the protein kinase and its substrates. Because our analysis reveals worms to have a mature P.Tyr-signaling network, especially

when compared with the yeast genome, we surveyed the worm genome for protein-tyrosine phosphatases.

Our analysis reveals 83 conventional protein-tyrosine phosphatases (PTPs) plus 6 catalytic fragments and 12 additional fragments with high homology to the noncatalytic portion of other worm PTPs. In addition, there are 26 proteins classified as dual-specificity phosphatases related to MAPK phosphatases, CDC14, PRL, PIR1, CDC25, myotubularins, or PTEN lipid phosphatases. We also identify two SBF1- and one STYX-related proteins that are related to myotubularins and MAPK phosphatases yet lack the catalytic cysteine motif. These proteins are predicted to be catalytically inert yet may function as phosphoprotein-binding domains or anti-phosphatases (36). We also identify 11 inositol polyphosphate phosphatases and 65 serine-threonine phosphatases. Among the 83 PTPs, there are 57 cytoplasmic PTPs and 26 receptor-like PTPs, most of which are worm specific, lacking clear human orthologues. Exceptions include worm orthologues of the cytoplasmic PTPs; SHP2, MEG1, and MEG2, and the receptor PTPs; and PTPδ, PTPγ, PTPμ, PTPβ and IA2 (catalytically inactive). Overall, worms contain approximately the same number of tyrosine and dual-specificity protein kinases as they do tyrosine and dual-specificity protein phosphatases. This coordinate expansion in the eukaryotic lineage of both protein-tyrosine kinases and phosphatases emphasizes the biological need to maintain tight regulation of tyrosine phosphorylation. Because of the large numbers of worm-specific PTKs (FER and KIN-15 families) and worm-specific PTPs (89%, or 66 of 74), it is tempting to speculate that these unique enzymes may regulate each other's activity, or function in the same signaling pathways. Precedence for such specificity comes from genetic data indicating that the CLR-1 receptor PTP attenuates EGL-15, an FGFR orthologue, signaling in worms (37).

Conclusions. What does the worm genome sequence tell us about mammalian signal transduction? First, it has provided an ideal model to highlight the bioinformatics challenges that lie ahead with the human genome effort and allows us to test our analysis tools and database organization. Second, it lets us refine our expectations as to the diversity and absolute number of unique protein kinases that we can expect to find in the human genome. Based on our count of 493 (411 conventional and 82 PK-like proteins) worm kinases, minus the ≈197 kinases that appear to be worm-specific expansions of certain families such as the CK1, FER, and KIN-15 families, multiplied by the ≈4-fold greater number of genes in humans compared with worms, we predict the human genome to contain ≈1,100 protein kinases (PTKs and serine/threonine kinases). A similar extrapolation predicts ≈300 human protein phosphatases (PTPs, dual-specificity phosphatases, and serine-threonine phosphatases). Because our current count of human protein kinases and phosphatases stands at ≈600 and 130, respectively, we still have about half the work ahead of us. However, recent claims predict the human genome may contain as many as 140,000 genes, compared with previous estimates of ≈80,000. Such calculations would result in a significant increase in our predictions of the total number of human protein kinases and phosphatases.

We may expect to see less evolutionary expansion of protein kinases families that serve elemental cellular functions such as cell cycle control and chromosome segregation, compared with processes involved in intercellular signaling or organogenesis. However, there is already evidence for at least a 2-fold expansion in the number of CDKs and "mitotic kinases" from worms to humans. Unlike expressed sequence tag data mining and PCR-based gene discovery approaches, genomic strategies do not bias against genes whose expression is tightly regulated in a cell-, developmental-, or disease-specific manner. This point is highlighted by the identification of 650 seven-transmembrane chemoreceptors in the worm genome (1), many of which may be expressed exclusively in single neurons. Because worms have only ≈302 neurons, compared with one trillion in humans, it would not be surprising to see this selectivity in cellular expression corroborated on mining the human genome. Indeed, because many of these novel protein kinases are likely to exhibit highly restricted expression, they may prove to be excellent targets for drug discovery in the battle against human disease.

The worm serves as a much simpler and tractable organism than humans for deciphering signaling cascades. Although their P.Tyr-signaling system is quite mature—based on the content of protein-tyrosine kinases, phosphatases, and adaptor molecules—they lack much of the molecular redundancy that exists in mammals, allowing the geneticist, biochemist, and cell biologist to more readily generate an "outline" of the signaling pathways that are conserved between worms and humans. The availability of the complete worm genome provides a unique opportunity to learn about human biology. Predicted orthologous pairs of human and worm genes can be targeted by using reverse genetic approaches to identify new signaling partners or biologic functions that can then be biochemically and functionally verified in mammals.

Although worms and humans have much in common, they also have obvious differences. Worms do not have limbs or bones, or a circulatory or immune system, and they eat only bacteria. Not surprisingly, they lack several protein kinases present in humans. Over the next 2 years, we should be better able to define which protein kinases are required for these specialized functions as the genome sequences of *Drosophila* and humans are completed. Identification and classification of the proteins present in each is just a first step toward understanding the biological complexity of life.

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