Serine/threonine protein kinases in *Drosophila*

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The study of serine/threonine kinases in Drosophila is coming of age. Recently several kinases have been identified and their role in cell determination has been established. This review discusses these recent findings and describes the potential for genetic analyses of kinase activity and signal transduction.

It has been more than 30 years since the first protein kinase was purified[1]. Since then more than 100 kinases have been identified in various organisms and this number probably represents just a subset of the total[2]. A great deal of information is available on the structure and biochemical properties of protein kinases, and it has become clear that they have multiple cellular functions in processes such as metabolism, cell cycle regulation, growth and differentiation[2,3]. In many cases, however, it has proven difficult to identify *in vivo* substrates and to characterize the mechanisms that regulate kinase activity. The identification of protein kinases in systems that are more amenable to genetic analyses now allows a dissection of specific pathways in which individual protein kinases are involved. In this review we describe recent findings in *Drosophila melanogaster* that focus on one class of protein kinases, those specific for phosphorylation of serine and threonine residues. By a combination of molecular and genetic approaches, several serine/threonine protein kinases have been identified in *Drosophila* and in some cases mutations are available. Analyses of these mutant phenotypes have established that protein kinases have roles in multiple developmental processes and suggest that they are major players in the establishment of cellular identity.

Properties of protein kinases

Protein kinases are a diverse family of enzymes capable of phosphorylating proteins at specific amino acids. A common feature of all protein kinases is the catalytic domain, which includes several highly conserved residues required for ATP binding and substrate phosphorylation[4]. Protein kinases can be broadly classified on the basis of their amino acid specificity: they phosphorylate either tyrosine or serine/threonine. The tyrosine protein kinase family falls into two groups: the growth factor receptor-like tyrosine kinases, which are transmembrane proteins, and the cytoplasmic or nuclear tyrosine kinases. Serine/threonine protein kinases are mostly cytoplasmic proteins; they play an important role in signal transduction and may serve as the cytoplasmic link between received extracellular signals and changes in cellular processes.

Serine/threonine protein kinases are generally classified by their mode of regulation. For example, there are the proteins that respond to second messages, such as the cyclic nucleotide-dependent kinases which require binding of cAMP or cGMP to stimulate kinase activity, and the protein kinase C class, which require calcium and phospholipids for enzyme activation. Another subfamily of serine/threonine protein kinases contains enzymes that are dependent on calcium and

TABLE 1. Serine/threonine protein kinases in *Drosophila*

Serine/threonine kinases	Chromosomal location	Phenotype	RNA distribution	References
Cyclic nucleotide-dependent				
cAMP-dependent				
DC0	30C	Unknown	Unknown	42
DC1	100A	Unknown	Unknown	42
DC2	72A	Unknown	Unknown	42
cGMP-dependent				
DG1	21D	Unknown	Unknown	42
DG2	24A	Unknown	Unknown	42
Protein kinase C				
dPKC	53E	Unknown	Unknown	43
dPKC53E	53E	Unknown	Photoreceptor cells	38
dPKC98F	98F	Unknown	Embryo and adult brain	38
Others				
casein	Unknown	Unknown	Unknown	44
D-Raf	2F6	Terminal class	Ubiquitous	21–23
ninaC	28A1-3	Electrophysiological response	Photoreceptor cells	36
zeste-white 3	3B1	Segment polarity	Ubiquitous	6, 7
fused	17C4-6	Segment polarity	Ubiquitous	19

In general these proteins have been identified as serine/threonine protein kinases on the basis of sequence homology, and they should therefore be considered putative protein kinases. During the isolation of cAMP- and cGMP-dependent kinases[41], six other cDNAs were isolated and demonstrated to be putative serine/threonine kinases but they were not dependent on cyclic nucleotides.

calmodulin. Finally, some serine/threonine kinases can be regulated by their state of phosphorylation. The various modes of regulation of these proteins reflect the diversity of the pathways in which they are involved. There is additional complexity at the level of substrate specificities: whereas some protein kinases have a single substrate – for example, the myosin light chain kinase phosphorylates only myosin light chain – other enzymes, such as protein kinase C, have multiple substrates[3].

Drosophila serine/threonine protein kinases

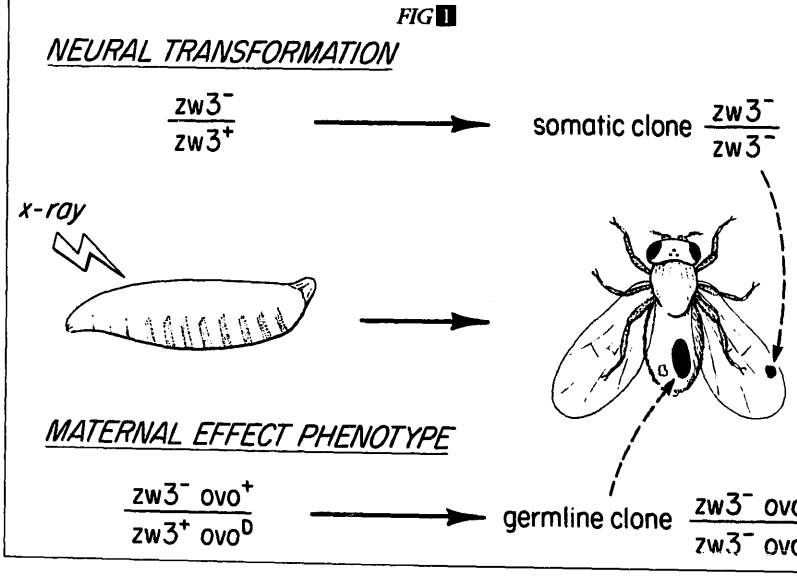
The study of protein kinases in *Drosophila* is a recent undertaking and therefore not as extensive as the study of mammalian kinases. However, the availability of mutants offers the potential to use genetics as a means to establish biochemical pathways, to identify potential substrates and to study the mechanisms that regulate activity. This type of analysis can be very powerful, as demonstrated by the elegant genetic studies of the control of cell cycle in the fission yeast, *Schizosaccharomyces pombe*, which have elucidated the role and regulation of the serine/threonine protein kinase $p34^{cdc2}$ (Ref. 5). In *Drosophila*, serine/threonine protein kinases have been identified either by their homology to the products of mammalian genes or as the products of genes initially isolated on the basis of their developmental mutant phenotypes (Table 1). To date, the serine/threonine kinases that have been identified by homology have not been linked to any mutant phenotypes. However, in several cases (the cyclic nucleotide-dependent kinases) a chromosomal location for the kinase gene has been identified, so it should be possible to screen for mutations in these genes. As for the protein kinases that have been identified on the basis of their mutant phenotype, the disruption of development indicates that they are involved in specific developmental decisions or cellular functions.

zeste-white 3

Several serine/threonine protein kinases have been described that are involved in processes that establish cell identity. One such serine/threonine kinase, encoded by the *zeste-white 3* (*zw3*) gene, is required for the establishment of cell fate at various times in the life cycle of the organism. The *zw3* gene encodes at least two distinct putative serine/threonine protein kinases that have identical catalytic domains but different amino termini. The proteins are developmentally regulated[6,7]: one is expressed maternally, the other in embryogenesis. There is a striking homology between the *zw3*-encoded kinases and a mammalian protein, glycogen synthase kinase-3/factor A (GSK-3)[8]. A comparison of the maternal *zw3* putative kinase and the predicted protein product of the β-class of GSK-3 cDNAs reveals an overall identity of 75% at the amino acid level and as much as 85% in the kinase catalytic domain (J. Woodgett, pers. commun.).

One zygotic role of *zw3* is the determination of epidermal versus neural fate among cells of the imaginal disc that give rise to the cuticle of the adult. The production of mosaics in imaginal discs (see Fig. 1) has demonstrated that patches of cells homozygous for *zw3* mutations, in an otherwise heterozygous tissue, will give rise to innervated ectopic bristles in the wing[9,10]. Cells that would normally give rise to epidermal cells are transformed to a neural fate. This transformation is also observed with mutations in the neurogenic genes[11]. The neurogenic loci are involved in lateral inhibition of the embryonic neuroectoderm, whereby individual cells will give rise to neuroblasts and inhibit the neighboring cells from assuming the neural fate. The same mechanism is probably used in the formation of adult sensory structures.

Lateral inhibition is likely to be mediated by a signal transduction pathway that links extracellular signals to changes in gene expression. Some of the products of the neurogenic genes are consistent with this interpretation: *Notch* and *Delta* encode transmembrane proteins with epidermal growth factor (EGF) repeats, and the products of the *Enhancer of split* complex include a putative G protein and DNA-binding proteins[12]. The putative serine/threonine protein kinase encoded by *zw3* may also play a role in the signal



The generation of germ-line and somatic clones by X-ray-induced mitotic recombination. Both germ-line and somatic clones are generated by irradiating a heterozygous mutant larva to induce a mitotic crossing-over event that will give rise to homozygous mutant cells in a heterozygous background. Mosaic germ lines are created by taking advantage of ovo^D, a dominant female-sterile mutation[35]. Cells that are of the genotype $zw3^{-}ovo^{+}/zw3^{+}ovo^{D}$ will not give rise to any functional ovaries so the only embryos that come from females of this genotype are the product of the homozygous mutant clones. This technique was also used to generate females with homozygous *l(1)pole-hole* germ-line clones to characterize the maternal requirement for *D-raf*.

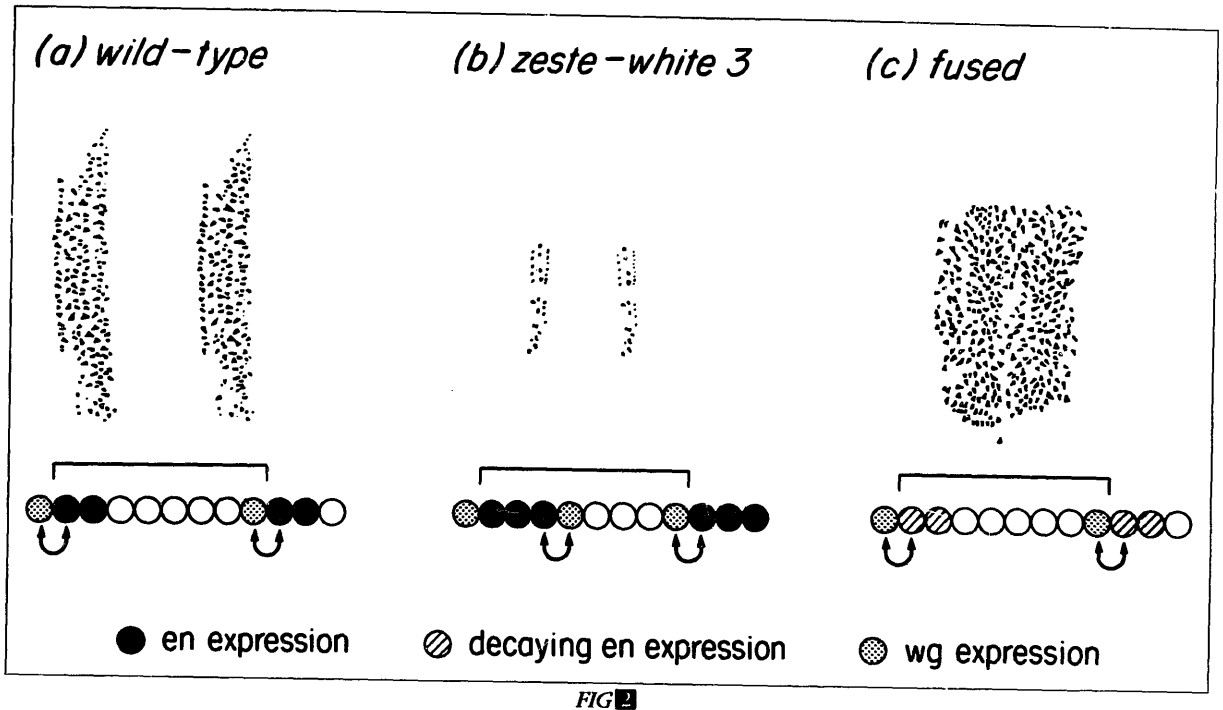


FIG 2

Serine/threonine protein kinases and intrasegmental patterning. The cuticle of each wild-type embryonic segment is covered by a belt of denticles anteriorly and is naked posteriorly. Mutations in any one of the segment polarity genes cause deletions of various cuticular structures, often accompanied by ectopic occurrence of others. Four classes of genes can be distinguished by their mutant phenotypes. The largest group, defined by the absence of posterior naked cuticle, includes *armadillo, dishevelled, porcupine, fused, wingless* (*wg*), *gooseberry, hedgehog, cubitus interruptusD* and *Cell*[10,20]. Mutations in the second group, *patched* and *costal-2*[18], cause deletion of more central pattern elements and the formation of extra segmental borders. Mutations in *engrailed* (*en*), the only known member of the third group, result in loss of the segmental borders. Finally, mutations in the fourth group, *naked* and *zeste-white 3* (*zw3*), cause the deletion of most denticles. The juxtaposition of the restricted domain of *wg* and *en* expression is required for the establishment of the parasegmental groove. The figure illustrates the cuticle phenotypes of (a) wild-type, (b) *zw3* and (c) *fu* embryos, and the corresponding cell states during gastrulation. The parasegmental unit is demarcated by a bracket. In *zw3* mutant embryos, ectopic *en* expression is detected posteriorly and an additional stripe of *wg* expression appears. In *fu* mutant embryos, both *wg* and *en* genes are initially expressed correctly; however, premature decay of *en* expression is observed, leading to cell death[15]. The extensive cell death in these mutant embryos plays a major role in the production of the terminal cuticular phenotype.

transduction pathway mediated by the neurogenic genes.

In addition to the imaginal function of *zw3*, maternally derived zeste-white 3 product, which is deposited in the unfertilized egg, is required for the establishment of correct embryonic segmentation. The *Drosophila* embryo is composed of repeating metameric units along the anteroposterior axis. The number, position and polarity of these units is dictated by the products of interacting maternal and zygotic segmentation genes[13]. The segment polarity genes, which include *zw3*, define the cell states that are required for proper organization of each segmental unit[14,15]. In wild-type embryos the juxtaposition of these different cell states leads to correct parasegmental boundaries and the differentiation of cuticular structures on the epidermis of the embryo (Fig. 2). A mutation in any one of the segment polarity genes results in incorrect cell specification and consequently a disruption of parasegmental boundaries and abnormal cuticle formation. Genetic analysis suggests that intercellular communication plays a central role in the expression of segment polarity genes and the refinement of cell state in each segment.

The molecular nature of some of the segment polarity genes is consistent with the notion that signal transduction is involved in the determination of cell identity within the segmental unit. The *wingless* (*wg*) gene encodes a secreted protein[16] that is homologous to the product of the mammalian proto-oncogene *int-1*. Another segment polarity gene, *patched*, encodes a transmembrane protein[17,18].

Homozygous *zw3* individuals die as larvae, so the maternal effect can only be examined in germ-line mosaics (see Fig. 1). Mutant *zw3* embryos derived from mosaic germ lines are devoid of most denticle hairs, a phenotype also produced by mutations in the zygotic segment polarity gene *naked* (*nkd*)[10]. One consequence of a mutation in any segment polarity gene is the disruption of the normal pattern of expression of other segment polarity genes, and indeed this is the case in both *nkd* and *zw3* mutant embryos (Refs 10, 14, and E. Siegfried *et al.* unpublished). In *zw3* and *nkd* mutant embryos the expression of *engrailed* (*en*) is expanded and there is an ectopic stripe of *wg* expression (Fig. 2). The similarity of mutant phenotypes and the nature of the product of the *zw3* gene suggest that the products of *zw3* and *nkd* interact directly. Further investigation of this interaction awaits the molecular characterization of *nkd*.

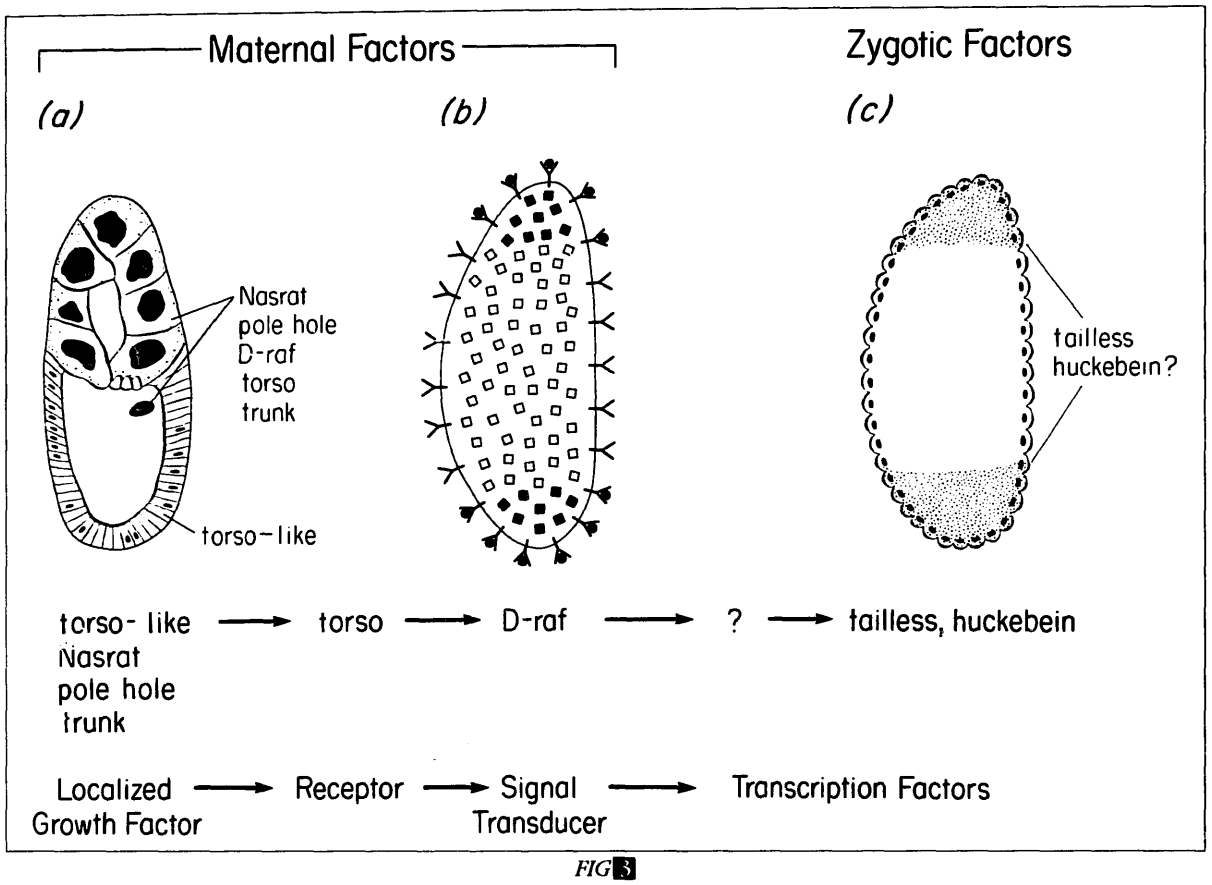


FIG 3

Serine/threonine protein kinases and the establishment of the embryonic fate map. The correct formation of the larva is dependent on the 'terminal class' genes. In their absence, an alteration of the embryonic fate map occurs, resulting in the truncation of the anterior head skeleton and absence of all structures posterior to the seventh abdominal segment. (a) This process requires proper germ-line expression of the *Nasrat*, *polehole*, *l(1)polehole* (*D-raf*), *torso* and *trunk* genes and expression of the *torso-like* gene in somatic follicle cells during oogenesis[24]. (b) Shortly after fertilization, the torso receptor associates with the embryonic membrane[27], and presumably is activated by binding to a spatially localized growth factor. The torso ligand may be the *torso-like* gene product secreted by the follicle cells located at the termini of the developing egg chamber[29]. In this model, activated torso stimulates D-raf kinase activity only at the termini. (c) Finally, activated D-raf affects transcriptional regulation of the zygotic genes *tailless* and *huckebein*. In this signal transduction pathway, it is unclear how D-raf acts to promote transcription. Y, torso protein; ●, torso ligand; □, D-raf protein; ■, activated D-raf protein.

fused

The *fused* (*fu*) gene is another maternal effect segment polarity gene that encodes a serine/threonine protein kinase[19]. Some *fu* mutations are homozygous viable, so the maternal effect can be examined in the progeny of *fu* mutant females. Although these individuals are viable, they do have deletions of various adult structures, indicating that *fu* is required in imaginal development. The *fu* mutant embryonic phenotype arises from the loss of naked cuticle in each segment, resulting in a lawn of denticles[20]. In *fu* mutant embryos expression of *en* decays prematurely; a similar decay is observed in *wg* mutants (Fig. 2)[15]. Although the initial establishment of *en* expression is controlled by the pair rule genes, maintenance of *en* expression requires the secreted product of the *wg* gene. The putative serine/threonine protein kinase encoded by *fu* may be involved in either the secretion or the reception of this *wg*-encoded signal.

l(1)polehole or *D-raf*

The *Drosophila-raf* (*D-raf*) gene provides a striking example of how a serine/threonine protein kinase acts to specify cell fates during embryonic development. The *D-raf* gene shows 45% homology at the amino acid level to the human *Raf-1* gene, with as much as 65% homology in the kinase domain[21,22] suggesting that these proteins will show similar mechanisms of regulation and substrate specificities. Mutations of the *l(1)polehole* gene have been shown by molecular analysis to disrupt the *D-raf* transcription unit, thus making a genetic analysis of *D-raf* possible[22,23].

Wild-type *D-raf* activity is required maternally for the normal establishment of cell fates along the anteroposterior axis of the embryo. During embryogenesis, specification of the acron (head) and telson (tail) cellular fates requires at least six maternal and two zygotic products[24]. The current model (Fig. 3) is that the maternal genes act in a signal transduction pathway to initiate transcription of the zygotic terminal gap genes at the embryonic poles. Genetic epistasis experiments have shown that all the maternal genes in this pathway act upstream of *torso*, with the exception of *D-raf*[23,25–27]. The *torso* gene encodes a putative transmembrane receptor tyrosine kinase[28] and neither its RNA nor its protein products are spatially restricted to the embryonic poles[27,28]. Receptor tyrosine kinases are activated when they bind to their ligands and transmit

this extracellular signal to the cytoplasm by phosphorylating intracellular proteins. It has been proposed that the *torso-like* gene product, shown by mosaic analysis to function in the follicle cell epithelium during oogenesis, may produce the putative torso ligand[29]. Local activation of torso by torso-like would activate a phosphorylation cascade mediated through D-raf, ultimately affecting the transcriptional regulation of the zygotic genes *tailless*[30] and *huckebein*[31]. Indeed, it has recently been shown that initiation of *tailless* expression is restricted to nuclei at the embryonic poles[30]. The *tailless* gene product, a member of the steroid receptor superfamily, may then function as a transcription factor to activate terminal-specific genes.

The proposed role of *D-raf* in the terminal class signal transduction pathway is in agreement with the conclusions reached for the function of *Raf-1* in mammalian cells. Results from biochemical studies indicate that the Raf-1 protein acts downstream of membrane-bound receptor tyrosine kinases. After cells are stimulated with growth factors such as platelet-derived growth factor (PDGF), or are transformed by membrane-associated oncogene products, the protein kinase activity of Raf-1 increases. This increase is associated with phosphorylation of Raf-1, primarily on serine and threonine residues, although a subpopulation of Raf-1 molecules is also phosphorylated on tyrosine in response to PDGF or transformation by v-*src*[32]. Direct association between Raf-1 and the activated PDGF receptor was demonstrated *in vitro*[33].

From the genetic analysis of mutations at the *l(1)polehole* locus it is clear that *D-raf* function is not restricted to the determination of the embryonic anteroposterior axis. Embryos lacking maternal and zygotic *D-raf* show, in addition to the deletion of terminal cell fates, massive epidermal cell death[34]. *D-raf* is also required zygotically for the control of somatic cell proliferation, since larvae that have no zygotic *D-raf* activity show atrophic imaginal discs[35].

ninaC

In contrast to serine/threonine kinases that are involved in establishment of cell fate, mutations at the *ninaC* locus illustrate an example of a cell-specific serine/threonine protein kinase involved in terminal differentiation of photoreceptor cells in the eye. *ninaC* mutant flies show abnormal electrophysiological responses, a result of reduced rhodopsin content. In such mutants, there is a reduction in the diameter of the rhabdomeres – specialized photoreceptor cell plasma membranes containing rhodopsin and other components of the phototransduction cascade. The *ninaC* gene encodes two hybrid cytoskeletal proteins that are composed of a putative serine/threonine kinase domain joined to a domain that resembles the myosin heavy chain gene[36]. Transcripts encoding both proteins are detected predominantly in the rhabdomeres of the photoreceptor cells. These kinase/myosin proteins may play a role in maintaining the structural integrity of the rhabdomeres or regulating proteins important for phototransduction. The distinctive structure of these proteins indicates that they may be associated with other cytoskeletal proteins. An interesting, somewhat analogous hybrid kinase has been described in *Caenorhabditis elegans*[37]. The *unc-22* gene encodes a muscle-specific protein, twitchin, that consists of a protein kinase domain similar to myosin light chain kinase, and several other motifs that are also found in the immunoglobulin superfamily and cell adhesion molecules. Genetic evidence and the structure of the protein are consistent with the possibility that twitchin interacts with other cytoskeletal proteins.

In addition to the cell-specific kinase/myosin proteins encoded by *ninaC*, there is evidence for an eye-specific protein kinase C in *Drosophila*. Among the three protein kinase C genes identified (Table 1), one is expressed exclusively in photoreceptor cells that have begun their terminal differentiation[38]. At present there are no mutations associated with any of these protein kinase C genes.

Perspectives

We have described several *Drosophila* serine/threonine protein kinases for which mutations are available and mutant phenotypes have been observed. The analysis of mutant phenotypes has allowed the identification of specific developmental pathways in which these kinases function. This analysis can also be used to determine if a kinase has numerous roles in development or is restricted to one process. *D-raf*, *zw3* and *fu* appear to function throughout the life cycle of the organism and may have multiple roles in development. This is in contrast to cell-specific kinases such as the *ninaC* gene product, which appear to be required only in photoreceptor cells.

The *ninaC* gene differs from these other kinase genes in a further respect: its role is in terminal differentiation, whereas *D-raf*, *zw3* and possibly *fu* are required for cell determination. Perhaps ninaC protein plays a role in maintaining the structural integrity of the rhabdomeres or in regulating proteins in the phototransduction cascade, both of which would only occur in the differentiated photoreceptor cell. *D-raf* and *zw3* appear to be involved in signal transduction pathways that serve to establish cell fate. How this change in cell fate is accomplished is unclear, but it is likely that both *zw3* and *D-raf* will affect the pattern of gene expression in undifferentiated cells.

Evidence for this comes from both genetic analyses of mutations that have similar mutant phenotypes to *l(1)polehole* (*D-raf*) and *zw3*, and from studies of homologous mammalian proteins. For example, a putative substrate for the *zw3*-encoded kinases is the product of the *nkd* gene, since mutations in these genes give a similar embryonic mutant phenotype. Alternatively, naked may act to regulate zeste-white 3 activity. At this time we cannot distinguish between the two possibilities, since the product of the *nkd* gene has not been characterized. The similarity between zeste-white 3 and the mammalian kinase GSK-3 may also provide some clues to possible substrates. GSK-3 has been implicated in the phosphorylation, and the inhibition of DNA-binding activity, of the product of the nuclear proto-oncogene c-*jun*[8]. The substantial similarity between GSK-3 and zeste-white 3 suggests that they may have similar substrate specificity; this hypothesis can be tested, since the *Drosophila* homologue of c-*jun* has recently been cloned[39,40].

D-raf may act to activate a transcription factor(s) by phosphorylation. This is suggested by genetic epistasis experiments, which place *D-raf* function downstream of the receptor tyrosine kinase gene *torso*, but upstream of the zygotic terminal genes. It is likely that *D-raf* is responsible for the activation of a transcription factor that in turn activates the expression of *tailless* and *huckebein* genes at the poles of the embryo (Fig. 3). At present the identity of this transcription factor is unknown. A similar role for Raf-1 is suggested by experiments in mammalian tissue culture cells, where Raf-1 acts as an intermediate in the regulation of transcription of the c-*fos* and actin genes[41].

In addition to the identification of substrates, it will be very important to establish how kinase activity is regulated. This can be achieved for *Drosophila* protein kinases through a combination of traditional biochemical and genetic approaches. Comparison with analogous mammalian systems will be important for studying the role of phosphorylation in kinase activity and the search for interacting proteins that may affect kinase activity. The availability of mutations in protein kinases allows one to screen for mutations that give similar phenotypes and may thus identify both regulators and substrates of kinase activity. In addition, it is possible to screen for mutations that either enhance or suppress the mutant kinase phenotype, and identify other proteins that interact with the kinase. For example, the proposal that D-raf activity is regulated by the tyrosine kinase receptor torso is supported by the analogous system of Raf-1 and PDGF receptor in mammals[33], and genetic epistasis experiments that have shown that *l(1)polehole* suppresses a gain-of-function *torso* mutation[23].

It is possible that differential regulation and/or different substrates accounts for the diversity of roles of kinases encoded by *zw3* and *l(1)polehole*. Both these genes are required maternally and zygotically, and function in the early embryo as well as having an imaginal function. As a consequence of this complexity, the production of somatic or germ-line mosaics (Fig. 1), in which patches of mutant cells are produced in an otherwise wild-type animal, may be the only way to detect the specific pathways in which these genes are operating. Through the use of mosaics and other genetic and biochemical approaches, the roles of serine/threonine protein kinases in signal transduction during *Drosophila* development can now be addressed. These studies are just beginning but they promise to be both exciting and fruitful in the years to come.

Acknowledgements

We thank M. Quinn for access to a protein kinase database, and T. Preat, P. Theron and J. Woodgett for unpublished data. Due to the limitation on the number of references we have cited reviews and a limited number of primary references. We apologize to authors whose work has not been cited. This work was supported by the Howard Hughes Medical Institute and an NIH grant to N.P. E.S. is supported by an NIH postdoctoral fellowship.

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