The Raf/MAP Kinase Cascade in Cell Cycle Regulation and Differentiation in Drosophila

Yasuyoshi Nishida1, Yoshihiro H. Inoue1, Leo Tsuda1, Takashi Adachi-Yamada1, Young-Mi Lim1, Mami Hata2, He-Yong Ha3, and Shin Sugiyama1

1Department of Biology, School of Science, Nagoya University, Chikusa-ku, Nagoya 464–01, Japan, 2Laboratory of Experimental Radiology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, Japan, and 3Department of Molecular Biology, College of Natural Science, Pusan National University, Pusan 609–735, Korea

Key words: signal transduction/terminal system/cell cycle

Regulation of cellular proliferation and differentiation during development are under strict control by spatially and temporally organized cell-to-cell interactions. Cellular signal transduction plays essential roles in these tremendously diverse and specialized processes. If the diversity and specificity of cell-to-cell signaling were correlated to factors specific to each process, a huge number of factors would be required and the genome would not be sufficient for the storage of the necessary genetic information. A variety of different signaling cascades and an increasing number of factors involved in signal transduction are being identified, and many of them have been demonstrated to function in multiple processes. The multiple use of factors or cascades would be reasonable for the economy of signal transduction, but it raises a question of how the specificity of each process is maintained while using common factors or cascades.

To fully understand the molecular mechanisms of development, it is crucial to unravel the cascades or networks of cellular signal transduction in a complete organism. Drosophila provides us an excellent model system for this as its genetics, molecular biology and developmental biology are well advanced. In this review, we describe the multi-functional nature of the highly conserved Raf/MAP kinase cascade in the regulation of cellular proliferation and differentiation in Drosophila.

Structure and Functions of D-raf

D-raf is unique in the Drosophila genome. Raf, a cytoplasmic serine/threonine protein kinase, plays essential roles in the transduction of a wide variety of growth-stimulating transmembrane signals (19, 31), and threeraf genes, c-raf, A-raf, and B-raf, comprise a gene family in mammals. In contrast to mammals, Drosophila carries a singleraf gene, D-raf, in its genome (22). The amino acid sequence of D-raf predicted from nucleotide sequences shows similar levels of identity to all the human Raf family members, suggesting that the multiplication and diversification of the raf genes took place in vertebrates after divergence from the invertebrate stock. The similarities are mostly confined to the regions, CR1, CR2 and CR3, which are conserved among the mammalian Raf family members (21, 22, 37). CR3 is the most conserved and corresponds to the kinase domain. The other conserved regions, CR1 and CR2, are assigned to the putative regulatory regions. While human Raf-1 has only 648 amino acid residues, D-raf consists of 781 amino acid residues due to a long amino-terminal extension (21, 37).

Multiple functions of D-raf. Mutants defective for D-raf have been identified by P element-mediated rescue experiments (22), and were found to be allelic to l(l)pole hole (l(l)ph) (1, 27). Mutants with a null function for D-raf are lethal at early pupal stages and dissection of the mutant larvae has shown undergrowth of tissues with proliferating cells, suggesting a role for D-raf in cellular proliferation. A quantification of proliferation rates by clonal analysis, or the so-called twin spot analysis, (18, 30) demonstrated that the rate of proliferation in the null mutants is reduced about 40% as compared to normal, explaining the major mutant phenotypes (40). The role for D-raf in cell cycle regulation will be discussed later in more detail.

A large amount of maternal D-raf mRNA was found to be accumulated in the ooplasm and germline mosaic analysis (see below) which demonstrated that the maternal D-raf activity is essential for the development of both anterior and posterior end structures of the embryo (1, 2, 22, 27). The roles for D-raf in the development of embryonic terminal regions will also be described in more detail below. Analysis of the phenotypes of a temperature-sensitive (ts) mutant and a hypomorphic mutant demonstrated that D-raf is required during most of the developmental stages, functioning in the development of the compound eye, wing, the muscle or peripheral nervous system in the thorax necessary
for eclosion and in the determination of dorsoventral polarity in the ovarian follicle cells (5, 16, 21). These observations, thus, suggest multiple roles for D-raf in the regulation of both cellular proliferation and differentiation and an involvement of D-raf in multiple signal transduction pathways.

Downstream Factors of D-raf

Genetic screening for downstream factors of D-raf. A powerful technique in genetics for the identification of novel factors involved in biochemical pathways such as signal transduction cascades is screening for suppressors or enhancers of mutations with known functions. For example, dominant gain-of-function mutations in genes encoding factors acting downstream of D-raf would be expected to suppress mutations of D-raf. Among about 200,000 flies screened, 18 mutations were associated with the dominant suppression of a hypomorphic mutation D-rafcuo carrying a point mutation causing an alteration of Arg-217 in the CR1 region to Leu (16, 21, 40). All of these suppressor mutations significantly rescued the defects in D-rafcno including eye development and wing vein formation and the lethality due to failure of eclosion (40; Lim et al., manuscript in preparation). Four of the mutations were mapped to the D-raf locus, and nucleotide sequencing of the mutant D-raf genes showed intragenic second site mutations leaving the original D-rafcno mutation intact (Lim et al., manuscript in preparation). A genetic analysis of the remaining mutations identified at least three loci outside D-raf: one on the X chromosome (Dsor1, Downstream suppressor of raf 1; eight alleles), one on the second chromosome (Dsor2; two alleles), and one on the third chromosome (Dsor3; five alleles) (40; Tsuda et al., manuscript in preparation).

Dsor1 encodes MAP kinase kinase. None of the suppressor mutations outside the D-raf locus rescued the viability of the null D-raf mutants, but detailed phenotypic analyses demonstrated a significant suppression of the mutant phenotype. In the dominant gain-of-function mutations of Dsor1 the reduced rates of proliferation due to the D-raf mutation recovered significantly. Dominant mutations of Dsor1 also partially rescued the posterior defect in embryos lacking maternal D-raf (40). If Dsor1 actually acts downstream of D-raf, it would be expected that its loss-of-function mutations would be associated with phenotypes similar to those of D-raf. Isolation of such mutants by screening revertants of the dominant Dsor1 mutation indicated that this was indeed the case: the mutants died at early pupal stages, showed severe defects in the tissues with proliferating cells inside a normal-looking larval body and had maternal effects on the development of the embryonic termini (40). The above genetic characterization of the Dsor1 mutations strongly indicates that this gene encodes a factor acting downstream of D-raf. Molecular cloning of Dsor1 by molecular tagging with the P element, a Drosophila transposon, demonstrated that it encodes a pro-

---

Fig. 1. A cassette of signal transduction. A signaling cascade composed of drk, Sos, Ras1, D-raf, Dsor1 and rolled mediate signals from multiple receptor tyrosine kinases such as sevenless, torso and DER as a cassette.
tein kinase similar to mitogen-activated protein kinase kinase (MAP kinase kinase or MAPKK) and indeed functions downstream of D-raf (40).

Dsor2 encodes MAP kinase. The dominant Dsor2 mutations were genetically mapped extremely close to the centromere of the second chromosome, where the rolled (rl) locus encoding the Drosophila homolog of MAP kinase (MAPK or DmERK-A) is located (4, 7). Nucleotide sequences of cDNAs for rl obtained by RT-PCR from flies homozygous for either of the two dominant mutations of Dsor2 showed alteration of single highly conserved amino acid residues in the kinase domain in each case (Lim et al., manuscript in preparation). The results strongly suggest that Dsor2 is allelic to rl and encodes the Drosophila homolog of MAP kinase. Molecular characterization of Dsor3 is currently underway. These observations demonstrated the presence of a highly conserved cascade of protein kinases composed of Raf, MAP kinase kinase and MAP kinase in Drosophila. The cascade functions downstream of multiple receptor tyrosine kinases as a part of a common cassette of signal transduction (Fig. 1) (9, 11, 17).

Cell Cycle-Specificities of D-raf and Dsor1

Cell cycle regulation during Drosophila development. Studies on the roles for Raf-1 and MAP kinase in cell proliferation have concentrated on the transduction of mitogenic transmembrane signals from growth factor receptors in the G0/G1 transition and G1 phase (3, 19, 31). It has been reported that MAP kinases are also activated in M phase (39). The following highly specialized features of cell cycle regulation during Drosophila development provide an excellent system to analyze the roles of these signaling factors in the cell cycle.

Four types of cell cycles are observed during Drosophila development (Fig. 2) (14). After fertilization, the 13 rapid and globally synchronous cycles of proliferation with no gap phases take place without division of the cytoplasm. Most of the nuclei migrate to the periphery of the embryo during this cleavage division stage to form a syncytial blastoderm. The plasma membrane grows rapidly between the peripheral nuclei during cycle 14, and the embryo develops into the cellular blastoderm. These individualized cells in cycle 14 become differentially regulated in the G2 phase, and most of the cells undergo three cycles of G2-regulated proliferation,
excluding the aminoserosa cells which are arrested in G2 of cycle 14. After cycle 16, the cells arrest in G1 phase for the remainder of embryonic development, except for the neuroblast cells which undergo further cycles of proliferation with G1, S, G2 and M phases. After hatching, the undifferentiated imaginal cells and germ cells proliferate extensively through G1-regulated cycles, while most of the differentiated larval cells rapidly grow by endocycles. In general, Drosophila cells undergoing terminal differentiation grow by endocycles in which cycles of DNA synthesis occur without mitosis. Thus, extensive cycles of endoreplication result in the formation of polytene chromosomes, typically observed in the larval salivary gland cells. After putation, the imaginal cells start their differentiation programs and now enter endocycles consisting of only S and G phases. Some of the nuclei remaining in the center of the early embryo, the yolk nuclei, and the nurse cells of germline origin in the adult ovary also undergo endocycles.

Roles in G1-regulated cycles. The functions of D-raf and Dsorl in cell cycle regulation can be assessed by analyzing the effects of their mutations on different types of cycles. As described above, mutants with a null function of either D-raf or Dsorl develop into superficially normal-looking larvae which, however, show severe undergrowth of internal tissues containing imaginal cells or germ cells (22, 40). These mutant phenotypes indicate that both D-raf and Dsorl are essential for the G1-regulated cycles of proliferation but not for the endocycles.

Roles in cleavage divisions. In these mutants, cell cycles during embryonic development are apparently not affected. As early embryonic development depends on the maternal contribution, this may be due to the deposition of a large amount of normal maternal D-raf and Dsorl products derived from the heterozygous maternal genome in the ooplasm. The requirement for D-raf and Dsorl in cleavage divisions can be analyzed by removing their maternal activities by the germline mosaic technique (26). Mitotic recombination induced by X-irradiation during the early larval stages of animals trans-heterozygous between null mutations for D-raf or Dsorl and Fs(l)ovoD1 produces germline clones, which are homozygous for D-raf or Dsorl but are devoid of Fs(l)ovoD1. Fs(l)ovoD1 is a dominant female-sterile mutation which prevents germ cells either heterozygous or homozygous for this mutation from maturing. Clones of cells homozygous for the D-raf or Dsorl mutation and devoid of Fs(l)ovoD1 produce mature oocytes lacking maternal D-raf or Dsorl function. Examination of the embryos defective for maternal D-raf after staining with Hoechst 33258 revealed no obvious aberration of cleavage divisions, suggesting that D-raf is not required for the free-run cycles composed of only S and M phases. On the other hand, mild but significant aberrations were observed in the cleavage stage embryos defective for maternal Dsorl: development is arrested during the early syncytial cleavage cycles in M phase in several to about 10% of the embryos (our unpublished observations). This suggests a role for Dsorl in M phase in the syncytial division cycles in addition to its role in the G1-regulated cycles.

D-raf may be G1-specific. Whether D-raf is also dispensable in M phase of the G1-regulated cycles was analyzed by using a temperature-sensitive (ts) mutation for D-raf. About 1.7% of the cells in the larval brain lobes are in M phase, and this fraction was not affected in wild-type larvae by shifting up the temperature under which they were raised. In contrast, the fraction of the M phase cells in the ts mutant decreased after transfer of the larvae to a restrictive temperature (16). This indicates that the mutant cells go through M phase normally but are arrested outside of M phase at the restrictive temperature. Thus, it can be said that D-raf is dispensable for the progression of M phase. Temperature-

![Fig. 3. Roles of D-raf and Dsorl in the cell cycle. Analyses of the cell cycle specificities of D-raf and Dsorl during development suggest that D-raf is a G1-specific activator of Dsorl. In contrast to D-raf, Dsorl may also function in M phase and may be regulated by an unknown activator in M phase.]
shifts during early embryonic stages also demonstrated no obvious aberrations during cleavage divisions and the G2-regulated cycles (our unpublished observations). These observations suggest that D-raf is a G1-specific activator of Dsorl and that Dsorl may be activated by a yet unknown upstream factor in M phase (Fig. 3).

**Network of Signal Transduction**

A cassette of signal transduction. Taking advantage of *Drosophila* genetics, the cascades transducing developmental signals from receptor tyrosine kinases, such as sevenless, torso and DER (*Drosophila* EGF receptor) have been extensively studied. These studies revealed that the transmembrane signals are transduced into the nucleus through a common cascade composed of drk (a SH3-SH2-SH3 adaptor molecular homologous to Grb2/Ash), sos, ras1, D-raf, Dsorl (MAP kinase) and rolled (MAP kinase) (Fig. 2) (9, 11, 17, 24, 33, 40; for reviews, see: 12, 15, 25). This suggests that mechanisms which give diversity and specificity to the cascade must exist. The torso receptor system presents a model system for investigating such problems.

**Terminal system.** The anteroposterior axis of the *Drosophila* embryo is determined by three independent systems known as the anterior, posterior and terminal systems (23). The anterior system determines the head and thorax, the posterior system determines the abdomin al regions, and the terminal system is responsible for the formation of the nonsegmented anterior and posterior or termini of the embryo. The terminal system employs a signal transduction mechanism mediated by the torso (tor)-encoded receptor tyrosine kinase (36). Torso is a ubiquitous surface receptor and is activated locally by a diffusible ligand, most probably encoded by torso-like (tsl) and produced at the extracellular terminal regions of the embryo (8, 35, 38). The signal from the receptor is transduced by the common cascade (1, 11, 17, 40) and regulates the zygotic expression of tailless (til) and huckebein (hkb), which themselves encode transcription factors (6, 28).

*tailless* is expressed in a pattern in the blastoderm embryo largely consistent with the mutant phenotype: a posterior cap and an anterior-dorsal stripe (28). The pattern is altered in the same manner in embryos laid by the terminal class maternal effect mutants: the posterior cap is lost and the anterior-dorsal stripe is expanded anteriorly (40). This indicates that the terminal system regulates the expression of *tailless* positively at the posterior end but negatively in the anterior domain. The diversification of signals transduced through apparently identical cascades at each end of the embryo may be explained in part by the cooperation of the terminal and the anterior systems in the regulation of *tailless* in the anterior region (29). Binding sites of bicoid protein, the morphogen in the anterior system, were identified in the promoter region of *tailless* and could be crucial for the activation of *tailless* expression in the anterior stripe (20). It has been shown that the bicoid-dependent activation of transcription is down-regulated by the torso receptor-mediated signaling cascade possibly by phosphorylation of bicoid by the *rolled*-encoded MAP kinase (32). Thus, the difference in the transduction factors functioning at the termini of the cascade would represent one of the mechanisms for a diversification of signal transduction. The transcription factor(s) responsible for the activation of *tailless* and *huckebein* at the posterior end remains to be identified.

**torso-mediated signaling cascade.** Involvement of Drk, Sos, Ras1, D-raf and Dsorl in the transduction of determinative signals from the Torso receptor has been reported (1, 11, 17, 40). Involvement of Rolled downstream of Dsorl has also been demonstrated by the observation that the torso-suppressing activity of the dominant gain-of-function mutation of *Dsorl* can be suppressed by reducing the gene dosage of *rolled* (our unpublished results). The embryos derived from germline clones homozygous for null functional mutations of *D-raf* or *Dsorl* lack terminal structures, and resemble torso mutant embryos. In contrast, only partial loss of terminal structures was observed in the embryos defective for the maternal activities of Drk, Sos or Ras1 (17). Embryos derived from the germline clones homozygous for the hypomorphic mutation of *D-raf*, *D-raf<sup>fc10</sup>* showed no loss of the terminal structures (17). The mutation in *D-raf<sup>fc10</sup>* causes an alteration of Arg-217 in the CR1 region to Leu and abolishes the physical association of D-raf with Ras1 (13, 17). Presuming that this interaction is essential for the activation of D-raf by Ras1, the results suggest that D-raf can be activated by a Ras1-independent parallel pathway.

From these observations a model for a signaling cascade mediated by the Torso receptor tyrosine kinase can be currently drawn as shown in Fig. 4. But this model is yet too simplified as there remain at least several unanswered questions. If the major consequence of Torso receptor stimulation is the activation of MAP kinase, dominant mutations of *Dsorl* or *rolled* should be expected to suppress the terminal defects. Indeed, the dominant constitutive active mutation of *Dsorl*, *Dsorl<sup>sup</sup>* significantly rescued the posterior defects in the terminal class mutant embryos. Even though loss-of-function *Dsorl* mutations cause deletion of both ends of the embryo, indicating that Dsorl is an essential component of the terminal system at both ends, the dominant *Dsorl* mutation failed to rescue the anterior defects (40). As seven more dominant *Dsorl* mutations all showed only similar effects on the terminal class mutant embryos (our unpublished observations), the results may represent differences in the factors or unidentified parallel cascade(s) interacting with Dsorl in the an-
The Torso-mediated signaling cascade regulates target gene expression differently at both termini of the embryo. Torso is a ubiquitous cell surface receptor and is activated at both anterior and posterior termini of the embryo by a diffusible extra-embryonic ligand produced by cooperation among the products of torso-like, trunk and fs(1)pole hole. The transmembrane signal is transduced by the common cassette into the nucleus and regulates the expression of tailless positively at the posterior end but negatively in the anterior domain. bicoid may be responsible for the activation of tailless in the anterior stripe and the Torso-mediated cascade may negatively regulate Bicoid. The transcription factor responsible for the activation of tailless at the posterior end remains to be identified.

Conclusions
A cascade of protein kinases composed of D-raf, Dsor1 and Rolled together with upstream activators, Drk, Sos and Ras1, comprise a signaling cascade cassette transducing the transmembrane signals from multiple receptor tyrosine kinases. Despite the common use of the cassette, a response specific to each receptor system is produced. Analysis of the roles of the member of this cassette in the regulation of cell proliferation suggested that each of them may function differently in different phases of the cell cycle, and that the cascade is not simple (Fig. 3). Analyses of the Torso-mediated signaling cascade have demonstrated possible mechanisms for diversification of the signal by recruitment of different transcription factors and branching of the cascade. Further studies on the genetic interactions between the genes for signaling factors of the same cascade or of other related cascades and identification of novel factors are necessary to further our understanding of the molecular mechanisms of development.

To identify novel factors which give diversity and specificity to this main cascade of signal transduction, we are currently trying to screen for P-insertional mutations which can be either suppressed or enhanced by the dominant Dsorl or Dsor2 mutations. So far we have obtained seven such mutations and each of them is associated with specific phenotypes affecting cell proliferation or adult morphologies. Molecular cloning of some of them has identified novel factors which may interact with the Raf/MAPK cascade to provide the specificity observed in signal transduction (our unpublished observations). Further systematic studies on such genes are anticipated to be useful in revealing the networks of signal transduction during development.

Acknowledgements. We are grateful to T. Tsuboi, K. Nishizawa, S. Tokumasu, S. Kawashima and S. Kachi for their technical assistance. This work was supported by grants from the Ministry of Education, Science and Culture of Japan.

REFERENCES
Drosophila Raf/MAP Kinase Cascade


