Genetic Analysis of *rolled*, Which Encodes a Drosophila Mitogen-Activated Protein Kinase

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ABSTRACT

Genetic and molecular characterization of the dominant suppressors of *D-raf*^{C110} on the second chromosome identified two gain-of-function alleles of *rolled* (*rl*), which encodes a mitogen-activated protein (MAP) kinase in Drosophila. One of the alleles, *rl*^{Su23}, was found to bear the same molecular lesion as *rl*^{Sem}, which has been reported to be dominant female sterile. However, *rl*^{Su23} and the current stock of *rl*^{Sem} showed only a weak dominant female sterility. Detailed analyses of the *rl* mutations demonstrated moderate dominant activities of these alleles in the Torso (Tor) signaling pathway, which explains the weak dominant female sterility observed in this study. The dominant *rl* mutations failed to suppress the terminal class maternal-effect mutations, suggesting that activation of Rl is essential, but not sufficient, for Tor signaling. Involvement of *rl* in cell proliferation was also demonstrated by clonal analysis. Branching and integration of signals in the MAP kinase cascade is discussed.

MITOGEN-activated protein kinase (MAP kinase or MAPK) plays essential roles in the transduction of diverse extracellular signals regulating cellular proliferation and differentiation. Its activity is closely regulated by phosphorylation of both threonine and tyrosine residues in its activation loop by a dual-specificity kinase MAP kinase kinase (MAPKK or MEK). MAPKK is also regulated by phosphorylation of two adjacent serine/threonine residues in its activation loop by MAP kinase kinase kinase (MAPKKK or MEKK). This cascade of protein kinases, known as the MAPK cascade, is highly conserved during evolution and found ubiquitously among eukaryotes (Nishida and Gotoh 1993; Davis 1994).

Drosophila also contains the MAPK cascade, and *rolled* (*rl*) (Biggs and Zipursky 1992; Biggs *et al.* 1994; Brunner *et al.* 1994), *Dsor1* (Tsuda *et al.* 1993; Lu *et al.* 1994), and *D-raf* (Nishida *et al.* 1988; Ambrosio *et al.* 1989) have been identified as encoding the components of the cascade, MAPK, MAPKK, and MAPKKK, respectively. Genetic analyses revealed the involvement of these genes in the transduction of signals from the receptor tyrosine kinases (RTKs) encoded by *sevenless* (*sev*), *torso* (*tor*), and Drosophila *EGF receptor homolog* (*DER*; Ambrosio *et al.* 1989; Dickson *et al.* 1992, 1996; Tsuda *et al.* 1993; Biggs *et al.* 1994; Brand and Perrimon 1994; Brunner *et al.* 1994; Hata *et al.* 1994; Hsu and Perrimon 1994; Nishida *et al.* 1996; Lim *et al.* 1997). The RTKs encoded by *sev* and *tor* are responsible for the determination of the R7 photoreceptor cell fate in the eye disc and of the cell fates at the embryonic termini, respectively (Hafen *et al.* 1993; Duffy and Perrimon 1994). *DER* has multiple functions, such as the determination of dorso-ventral polarity of the ovarian follicle, the embryonic ectodermal differentiation, and the differentiation of the compound eye and wing veins (Shilo and Raz 1991). In addition, *D-raf* and *Dsor1* have been demonstrated to be involved in the regulation of cellular proliferation (Perrimon *et al.* 1985; Nishida *et al.* 1988, 1996; Tsuda *et al.* 1993; Hata *et al.* 1994). Thus, the ubiquitous MAPK cascade receives diverse extracellular signals and generates responses specific to each RTK. Elucidation of the molecular mechanism by which signal specificity is generated will be crucial for understanding the molecular mechanisms of development.

It has been demonstrated that the transduction of the signals generated by different RTKs is mediated through a cassette of pathways composed of multifunctional factors encoded by *drk*, *Sos*, *Ras1*, *D-raf*, *Dsor1*, and *rl* (Ambrosio *et al.* 1989; Doyle and Bishop 1993; Lu *et al.* 1993a; Tsuda *et al.* 1993; Brunner *et al.* 1994; Diaz-Benjumea and Hafen 1994). However, the cassette does not seem to be a simple, straightforward pathway, and the signal specificity in each RTK pathway may be provided by branching and integration of signals as well as

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the differential modulation of some components. For example, it has been proposed that a Ras1-independent pathway activates D-raf in the Tor pathway from the observation that a loss of *Ras1* activity did not completely abolish Tor signaling (Hou *et al.* 1995). Genetic analysis of gain-of-function mutations of *Dsor1* demonstrated that Dsor1 transduces signals less efficiently in the DER pathway than in the Tor or Sev pathways, suggesting that differential modulation of the Dsor1 activity may be involved in generating signal specificity (Lim *et al.* 1997). We herein characterize gain-of-function mutations of *rl* and their activities in Tor signaling and in imaginal cell proliferation. The results obtained in this study suggest novel points for the integration or branching of signals in the MAPK cascade.

MATERIALS AND METHODS

Genetics: Fly cultures and crosses were performed at 25° unless otherwise described. Fly stocks used in this study were provided as follows: $fs(1)ph^{1901}/FM6$ and tor^{RL3} *cn px sp*/*CyO* from G. Struhl; $D\text{-}raf^{C110}$; rl^{Sem} from D. Brunner and E. Hafen; *w*; $l(2)rl^{EMS64}$ *Pin*/*SM1* from D. Yamamoto; and rl^{1}, $Df(2R)rl^{10a}$, *lt* rl^{10a} *cn*/*SM1* and *Df(2R)* rl^{10b}, *lt* rl^{10b} *cn*/*SM1* from the Bloomington Stock Center. For descriptions of the genetic markers and balancers, see Lindsley and Zimm (1992). $D\text{-}raf^{C110}$/Y; rl^{Sem}/rl^{Sem} males were crossed with Canton-S (wild-type) females and their male progeny were further crossed with Canton-S females to remove $D\text{-}raf^{C110}$. Females heterozygous for rl^{Sem} (+/+;rl^{Sem}/+) were selected by the dominant eye/wing phenotypes and tested for dominant sterility.

Clonal or twin-spot analysis was performed with *Dp(1; Y; 3)M2′*, mwh^{+} ve^{+} *FR1*, *y cv v* $f^{36a}/C(1)RX$, *y f*/$B^{S}Y$; *mwh ve h* as described earlier (Lawrence *et al.* 1986; Tsuda *et al.* 1993). Females with relevant mutations that were also homozygous for *mwh* were crossed with males of the above strain, and their progeny were irradiated with X ray (1500 R) during early third instar (~72 hr after eggs were laid). Clones formed in wing blades were analyzed, and only those twin-spots with >16 f^{36a} (control) cells were considered.

Molecular procedures: RNA was extracted from homogenized adult flies homozygous for either rl^{Su14} or rl^{Su23}, and cDNAs were synthesized using oligo(dT) primers and Superscript reverse transcriptase (Boehringer, Indianapolis). The mutant *rl* cDNAs were cloned as two overlapping fragments synthesized by RT-PCR using sets of sense and antisense primers synthesized according to the *rl* coding sequence (Biggs and Zipursky 1992). The sense primers were 5′-GAGGATTC CGACAAGTGAATTTATTCTATTTCACCC-3′ and 5′-GAG GATTCCATGTCACAAACTACCTCAGAC-3′, and the antisense primers were 5′-GAGGATCCTCCCGATGCAAGACGT TTGCGGAATG-3′ and 5′-GAGGATTCGCAAAATGGAGAAG TCCAGC-3′. Each primer contained a *Bam*HI restriction site (underlined) to facilitate ligation into the *Bam*HI cloning site in pBluescript (Stratagene, La Jolla, CA). Three independent clones were sequenced on each strand using synthetic oligonucleotide primers.

Whole-mount *in situ* hybridizations: Digoxigenin-labeled antisense and sense RNA probes were made from linearized plasmid DNAs containing *tll*, *hkb*, or *ftz* cDNA fragments using the DIG RNA labeling kit (Boehringer). Probes were treated with alkali to reduce their sizes to an average of 100 nucleotides long and were used for *in situ* hybridization with whole-mount embryos following the method of Tautz and Pfeifle (1989), with slight modifications.

Other procedures: Histological sections for electron microscopy were prepared as previously described (Basler *et al.* 1991). Cuticle preparations of embryos were made as described by Wieschaus and Nüsslein-Volhard (1986), and viewed with dark-field optics.

RESULTS

Genetic and molecular characterization of gain-of-function mutations of *rolled*: To identify factors acting downstream of D-raf, we screened for dominant suppressors of a hypomorphic allele of *D-raf*, $D\text{-}raf^{C110}$, and obtained 19 such mutants (Tsuda *et al.* 1993; Lim *et al.* 1997). In this article, the two second chromosomal suppressors, *Su14* and *Su23*, are described. These mutations fully suppressed the phenotypes of $D\text{-}raf^{C110}$ in terms of the viability and the morphologies of the compound eye and wing veins (data not shown). Both mutations mapped genetically at 55.0 in the centromeric heterochromatic region of the second chromosome, where *rolled* (*rl*), encoding a Drosophila homolog of MAPK, is located (Biggs *et al.* 1994).

In the $D\text{-}raf^{+}$ background, *Su23* caused a dominant phenotype, producing a mild rough eye and extra wing veins (Figure 1B and Figure 2B). Observation of ultrathin sections of the compound eye revealed multiple R7-like cells in each ommatidium (Figure 1D). Flies heterozygous for *Su14* showed no apparent rough eye phenotype, but observations of their eye sections revealed extra R7-like cells in a small fraction of ommatidia. Flies homozygous for *Su14* showed a mild rough eye phenotype with multiple R7-like cells in most of the ommatidia (Figure 1E). Both mutations strongly suppressed the loss-of-function mutation of *sev*, and extra R7-like cells were produced in *Su23* even with the genetic background of a null *sev* mutation (data not shown).

A gain-of-function mutation of *rl*, $rl^{Sevenmaker}$ (rl^{Sem}) resulted in a similar phenotype that was significantly enhanced by loss-of-function mutations of *rl* (Brunner *et al.* 1994). We observed that *rl* mutations significantly enhanced the wing phenotype seen in *Su23*, and that more wing vein materials were produced in $Su23/rl^{1}$ than in *Su23*/+ (Figure 2D). The eye phenotype was also enhanced and more R7-like cells were produced in $Su23/rl^{1}$ and $Su23/Df(2R)rl^{10b}$ flies [3.19 ± 1.30 (N = 97) and 3.18 ± 1.23 (N = 90) R7-like cells per ommatidium, respectively; Figure 1F] than in *Su23*/+ flies (2.75 ± 1.33 R7-like cells per ommatidium, N = 122). Although *Su14*/+ showed no extra wing veins, flies transheterozygous for rl^{1} and *Su14* did (Figure 2C). The enhancement of the dominant activities may be due to lack of competition from the normal product, and the results may suggest that they are the alleles of *rl*.

To confirm the allelism further, we cloned the *rl* cDNA fragments by RT-PCR with template RNAs ex-

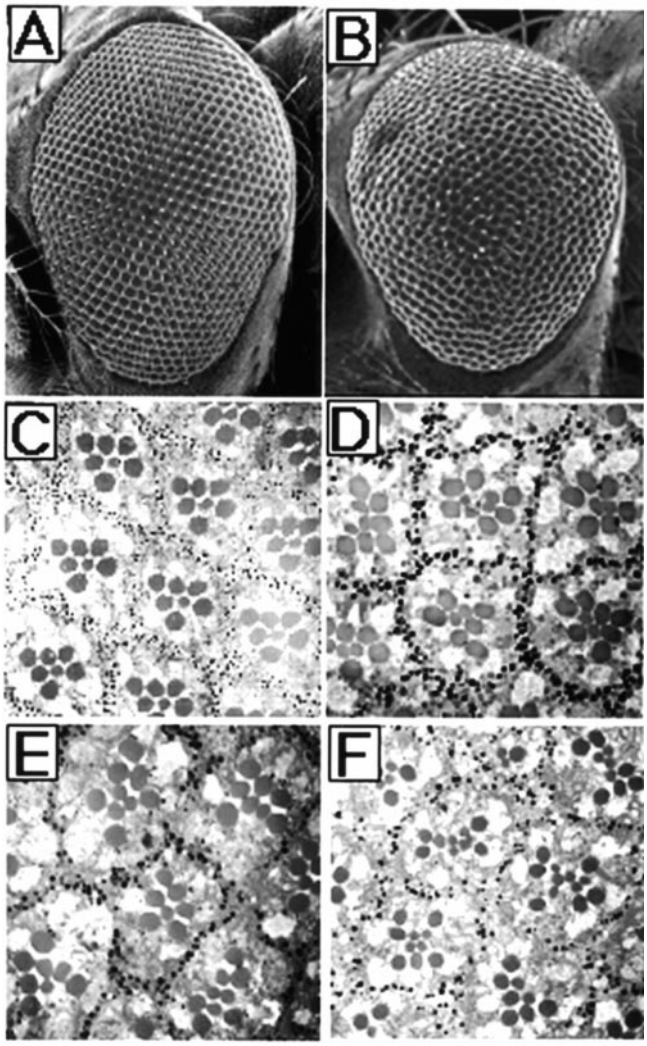

Figure 1.—Scanning (A and B) and transmission (C–F) electron microscopy of compound eyes. The compound eye of a wild-type fly is composed of a regular array of ommatidia (A). Within each ommatidium, a centrally located smaller rhabdomere derived from the R7 photoreceptor cell, and six large outer rhabdomeres from the outer photoreceptor cells (R1 to R6) with surrounding pigment cells, can be seen in a tangential section of the eye (C). In a $rl^{Su23}/+$ fly, the eye surface is rough (B) and each ommatidium contains multiple R7-like cells that are marked with small rhabdomeres (D). A rl^{Su14}/rl^{Su14} fly has a mild rough eye phenotype (not shown) with multiple R7-like cells formed in most of the ommatidia (E). The eye phenotype of $rl^{Su23}/+$ was significantly enhanced in $rl^{Su23}/Df(2R)rl^{10b}$ (F).

tracted from flies homozygous for the suppressor mutations, and sequenced them as described in materials and methods. Comparison of the nucleotide sequences with those reported (Biggs and Zipursky 1992) revealed guanine-to-adenine substitutions at nucleotide position 737 in *Su14* and 1214 in *Su23*, respectively, causing changes from aspartic acid to asparagine at amino acid residue 185 in kinase subdomain VII in *Su14* and at 334 in kinase subdomain XI in *Su23*, respectively (Figure 3). Both residues are highly conserved among MAPK family members, and the alterations are likely the cause of the suppressor mutations. Hereafter, we refer to them as rl^{Su14} and rl^{Su23}. To our surprise, the molecular lesion associated with rl^{Su23} was identical to that found in rl^{Sem} (Brunner *et al.* 1994).

Activity of *rolled* in the Torso pathway: It has been reported that flies heterozygous for rl^{Sem} are almost invariably dominant female sterile (Brunner *et al.* 1994). On the contrary, $rl^{Su23}/+$ females are fertile, and a detailed analysis revealed a weak dominant sterility. A fraction of the eggs (10.9%, $N = 2688$) laid by $rl^{Su23}/+$ females crossed with normal males failed to hatch, and observation of the cuticular pattern of the nonviable embryos revealed that 15.6% ($N = 205$) of them were associated with reduction in the number of abdominal segments (data not shown). This phenotype is similar to that observed in embryos produced by females carrying gain-of-function muations in *tor* (Klingler *et al.* 1988). The remaining nonviable embryos showed no obvious aberration. Reexamination of rl^{Sem}, kindly provided by D. Brunner and E. Hafen, also demonstrated a weak dominant sterility similar to rl^{Su23}. The $rl^{Sem}/+$ flies produced nonviable embryos (10.2%, $N = 2066$), a fraction of which (20.0%, $N = 184$) showed defects similar to those associated with the gain-of-function alleles of *tor*. Thus, both rl^{Su23} and the current stock of rl^{Sem} are only weakly dominant female sterile. Females homozygous for rl^{Su23} are essentially sterile due to defects in vitellogenesis (data not shown). Due to this discrepancy, we analyzed the effects of the *rl* mutations in the terminal system in more detail.

The effects of the gain-of-function mutations of *rl* on the expression of *tll* and *hkb*, target genes in the Tor signaling pathway (Pignoni *et al.* 1990; Weigel *et al.* 1990; Tsuda *et al.* 1993), were examined. In normal blastoderm embryos, *tll* is expressed in a pattern having a posterior cap and an anterior dorsal-lateral stripe (Pignoni *et al.* 1990; Tsuda *et al.* 1993; Figure 4A). In the embryos derived from the *tor* dominant females, the *tll* expression region is greatly expanded into the central region, and the expression pattern of the pair-rule gene *fushitarazu* (*ftz*) is severely affected so as to produce only three stripes instead of the normal seven stripes (Steingrimsson *et al.* 1991; Strecker *et al.* 1991). In the embryos laid by $rl^{Su23}/+$ or $rl^{Su14}/+$ females (Figure 4, B and C), no gross alteration of the *tll* expression pattern was observed, although the *tll*-expressing regions were significantly expanded. The anterior border of the posterior *tll* expression region was shifted to 19.3% egg length (EL) and 18.8% EL in the embryos laid by $rl^{Su23}/+$ or $rl^{Su14}/+$ females, respectively, as compared to 14.6% EL in normal embryos. There was also a significant posterior shift in the anterior border of the anterior stripe in these embryos (Figure 4, B and C). Another target gene, *hkb*, is expressed at both termini in blastoderm embryos (Weigel *et al.* 1990; Figure 4F), and its expression was also significantly expanded at

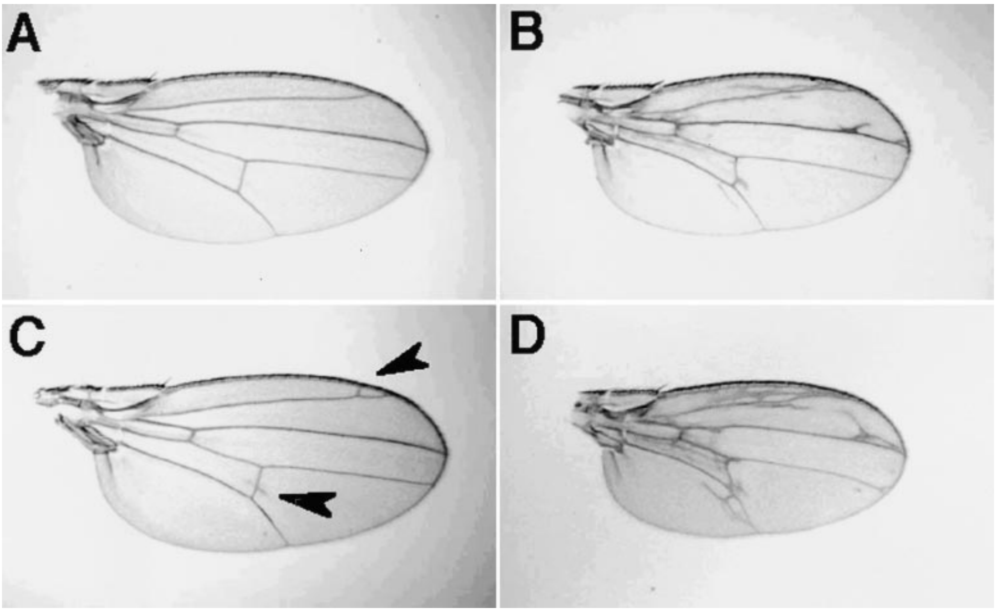

Figure 2.—Wing phenotypes with the gain-of-function mutations in *rl* and their enhancement by loss-of-function mutations in *rl*. (A) A wing blade of a wild-type fly. Extra wing vein materials are formed in the wing of $rl^{Su23}/+$ (B), and the phenotype was significantly enhanced in $rl^{Su23}/Df(2R)rl^{10b}$ (D). Wings of $rl^{Su14}/+$ are normal, but extra wing veins (arrowheads) were occasionally formed in $rl^{Su14}/Df(2R)rl^{10b}$ (C).

both termini in these embryos (Figure 4, G and H). No gross alteration of the expression pattern of *ftz* was observed, although the seven stripes were considerably condensed toward the central region (Figure 4L). As most of the embryos develop normally, the altered pattern of development would thus be regulated during later stages. Similar results were obtained with the embryos laid by $rl^{Sem}/+$ females (data not shown).

We also analyzed the genetic interactions of *rl* with the terminal class maternal-effect mutations. Embryos

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Dm Rl      MEEFNSSGSV VNGTG-STEV PQSNAEVIRG QIFEVGPRYI KLAYLGEGAY GMVVSADDTL TNQRVAIKKI SPFEHQTYCQ RTLREITILT RFKHENIIDI   99
h ERK1     PRRTEGVGP- ----G----V P-GEVEMVKG QPFDVGPRYT QLQYIGEGAY GMVSSAYDHV RKTRVAIKKI SPFEHQTYCQ RTLREIQILL RFRHENVIGI   90
h ERK2     MAAAAAAGA- ----G----- P----EMVRG QVFDVGPRYT NLSYIGEGAY GMVCSAYDNV NKVRVAIKKI SPFEHQTYCQ RTLREIKILL RFRHENIIGI   86
rat ERK2   MAAAAAAG-- ---------- P----EMVRG QVFDVGPRYT NLSYIGEGAY GMVCSAYDNL NKVRVAIKKI SPFEHQTYCQ RTLREIKILL RFRHENIIGI   84
Xl MAPK    MAAAGAA-S- -NP-GGG--- P----EMVRG QAFDVGPRYI NLAYIGEGAY GMVCSAHDNV NKVRVAIKKI SPFEHQTYCQ RTLREIKILL RFKHENIIGI   89
Sc FUS3    M--------- -----PKRIV YNISS----- ---D----FQ LKSLLGEGAY GVVCSATHKP TGEIVAIKKI EPFDKPLFAL RTLREIKILK HFKHENIITI   74
Sp spk1    MASATSTPTI ADGNSNKESV ATSRSPHTHD LNFELPEEYE MINLIGQGAY GVVCAALHKP SGLKVAVKKI HPFNHPVFCL RTLREIKLLR HFRHENIISI  100

                                                                                          N(Su14)
Dm Rl      RDILRVDSID QMRDVYIVQC LMETDLYKLL KTQRLSNDHI CYFLYQILRG LKYIHSANVL HRDLKPSNLL LNKTCDLKIC DFGLARIADP EHDHTGF---  196
h ERK1     RDILRASTLE AMRDVYIVQD LMETDLYKLL KSQQLSNDHI CYFLYQILRG LKYIHSANVL HRDLKPSNLL INTTCDLKIC DFGLARIADP EHDHTGF---  187
h ERK2     NDIIRAPTIE QMKDVYIVQD LMETDLYKLL KTQHLSNDHI CYFLYQILRG LKYIHSANVL HRDLKPSNLL LNTTCDLKIC DFGLARVADP DHDHTGF---  183
rat ERK2   NDIIRAPTIE QMKDVYIVQD LMETDLYKLL KTQHLSNDHI CYFLYQILRG LKYIHSANVL HRDLKPSNLL LNTTCDLKIC DFGLARVADP DHDHTGF---  171
Xl MAPK    NDIIRAPTIE QMKDVYIVQD LMETDLYKLL KTQHLSNDHI CYFLYQILRG LKYIHSANVL HRDLKPSNLL LNTTCDLKIC DFGLARVADP DHDHTGF---  186
Sc FUS3    FNIQRPDSFE NFNEVYIIQE LMQTDLHRVI STQMLSDDHI QYFIYQTLRA VKVLHGSNVI HRDLKPSNLL INSNCDLKVC DFGLARIIDE SAADNSEPTG  174
Sp spk1    LDILPPPSYQ ELEDVYIVQE LMETDLYRVI RSQPLSDDHC QYFTYQILRA LKAMHSAGVV HRDLKPSNLL LNANCDLKVA DFGLARSTTA QGGNPGF---  197

Dm Rl      ----LTEYVA TRWYRAPEIM LNSKGYTKSI DIWSVGCILA EMLSNRPIFP GKHYLDQLNH ILGVLGSP-S RDDLECIINE KARNYLESLP FKPNVPWAKL  291
h ERK1     ----LTEYVA TRWYRAPEIM LNSKGYTKSI DIWSVGCILA EMLSNRPIFP GKHYLDQLNH ILGILGSP-S QEDLNCIINM KARNYLQSLP SKTKVAWAKL  282
h ERK2     ----LTEYVA TRWYRAPEIM LNSKGYTKSI DIWSVGCILA EMLSNRPIFP GKHYLDQLNH ILGILGSP-S QEDLNCIINL KARNYLLSLP HKNKVPWNRL  278
rat ERK2   ----LTEYVA TRWYRAPEIM LNSKGYTKSI DIWSVGCILA EMLSNRPIFP GKHYLDQLNH ILGILGSP-S QEDLNCIINL KARNYLLSLP HKNKVPWNRL  266
Xl MAPK    ----LTEYVA TRWYRAPEIM LNSKGYTKSI DIWSVGCILA EMLSNRPIFP GKHYLDQLNH ILGILGSP-S QEDLNCIINL KARNYLLSLP HKNKVPWNRL  281
Sc FUS3    QQSGMTEYVA TRWYRAPEVM LTSAKYSRAM DVWSCGCILA ELFLRRPIFP GRDYRHQLLL IFGIIGTPHS DNDLRCIESP RAREYIKSLP MYPAAPLEKM  274
Sp spk1    ----MTEYVA TRWYRAPEIM LSFREYSKAI DLWSTGCILA EMLSARPLFP GKDYHSQITL ILNILGTP-T MDDFSRIKSA RARKYIKSLP FTPKVSFKAL  292

                                                      N(Su23)
Dm Rl      FPNADALALD LLGKMLTFNP HKRIPVEEAL AHPYLEQYYD PGDEPVAEVP FRINM EN-- --DDISRDAL KSLIFEETLK FKERQPDNAP             376
h ERK1     FPKSDSKALD LLDRMLTFNP NKRITVEEAL AHPYLEQYYD PTDEPVAEEP FTFAM EL-- --DDLPKERL KELIFQETAR FQPGVLE-AP             366
h ERK2     FPNADSKALD LLDKMLTFNP HKRIEVEQAL AHPYLEQYYD PSDEPIAEAP FKFDM EL-- --DDLPKEKL KELIFEETAR FQPGYRS                360
rat ERK2   FPNADSKALD LLDKMLTFNP HKRIEVEQAL AHPYLEQYYD PSDEPIAEAP FKFDM EL-- --DDLPKEKL KELIFEETAR FQPGYRS                358
Xl MAPK    FPNADPKALD LLDKMLTFNP HKRIEVEAAL AHPYLEQYYD PSDEPVAEAP FKFEM EL-- --DDLPKETL KELIFEETAR FQPGY                  361
Sc FUS3    FPRVNPKGID LLQRMLVFDP AKRITAKEAL EHPYLQTYHD PNDEPEGEPI PPSFF EFDH HKEALTTKDL KKLIWNEIFS                           353
Sp spk1    FPQASPDAID LLEKLLTFNP DKRITAEEAL KHPYVAAYHD ASDEPTASPM PPNLV DLYC NKEDLEIPVL KALIFREVN- FR                        372
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Figure 3.—An alignment of the amino acid sequence of Rl with its homologs and molecular lesions associated with rl^{Su14} and rl^{Su23}. The amino acid sequence of Rl (Dm-Rl; Biggs *et al.* 1994) was aligned with those of MAP kinases from human (h-ERK1 and h-ERK2; Owaki *et al.* 1992), rat (rat-ERK2; Boulton *et al.* 1991), Xenopus (Xl-MAPK; Gotoh *et al.* 1991), budding yeast (Sc-FUS3; Elion *et al.* 1990), and fission yeast (Sp-spk1; Toda *et al.* 1991). Dashes indicate insertions introduced to optimize similarities, and the residues identical to those of Rl are shaded. Alterations found in Rl^{Su14} and Rl^{Su23} are indicated with bold letters above the Rl sequence. A guanine-to-adenine alteration in each allele caused an amino acid substitution of aspartic acid to asparagine at residue 185 in Rl^{Su14} and at residue 334 in Rl^{Su23}.

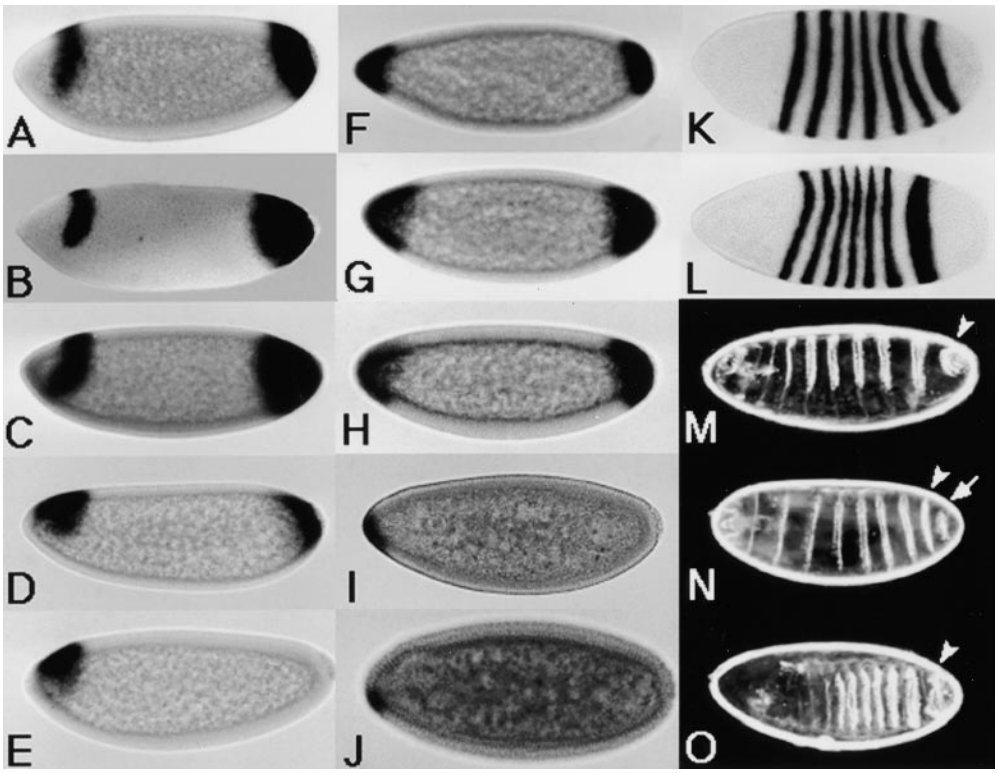

Figure 4.—The gain-of-function mutations of *rl* strengthened the signal from the Tor receptor tyrosine kinase but failed to suppress terminal class mutations. Expression patterns of *tll* (A–E), *hkb* (F–J), and *ftz* (K and L) in the cellular blastoderm-stage embryos laid by females with the following genotypes: Canton-S (normal) (A, F, and K), rl^{Su23}/+ (B, G, L), rl^{Su14}/+ (C and H), $fs(1)ph^{1901}$ $Dsor1^{Su1}$/$fs(1)ph^{1901}$ + (D and I), and $fs(1)ph^{1901}$/$fs(1)ph^{1901}$; rl^{Su23}/+ (E and J). Expression of *tll*, *hkb*, and *ftz* was visualized by *in situ* hybridization of whole-mount embryos (Tautz and Pfeifle 1989). Cuticular preparations of embryos (M–O). The terminal structures posterior to the seventh abdominal segment (arrowheads) are missing in the embryo laid by a $fs(1)ph^{1901}$/$fs(1)ph^{1901}$ female (M). The posterior defect was significantly suppressed in the embryo laid by a $fs(1)ph^{1901}$ $Dsor1^{Su1}$/$fs(1)ph^{1901}$ + female, and an eighth abdominal segment (arrow) was formed (N). On the other hand, the defect was preserved in the embryo laid by a $fs(1)ph^{1901}$/$fs(1)ph^{1901}$; rl^{Su23}/+ female (O).

produced by females homozygous for terminal class maternal-effect mutations such as *fs(1)ph* fail to develop structures posterior to the seventh abdominal segment as well as anterior-most structures, including the head skeleton (Figure 4M and Table 1). Expression of *tll* and *hkb* is severely affected in these embryos (Weigel *et al.* 1990; Tsuda *et al.* 1993; not shown, but essentially identical to expression shown in Figure 4, E and J).

TABLE 1

Effects of *rl* and *Dsor1* mutations on the posterior defects of embryos produced by females homozygous for $fs(1)ph^{1901}$

Maternal genotype	Embryos with A8[a] (%)	*N*
ph^{1901}/ph^{1901}; +/+	5.3	528
ph^{1901} $Dsor1^{Su1}$/ph^{1901}+; +/+	91.9	1863
ph^{1901} $Dsor1^{Su1}$/ph^{1901}+; rl^{EMS64}/+	76.8	1017
ph^{1901} $Dsor1^{Su1}$/ph^{1901}+; rl^{10a}/+	46.5	144
ph^{1901} $Dsor1^{Su1}$/ph^{1901}+; rl^{10b}/+	25.7	210
ph^{1901}/ph^{1901}; rl^{Su14}/+	5.3	888
ph^{1901}/ph^{1901}; rl^{Su14}/rl^{Su14}	4.9	286
ph^{1901}/ph^{1901}; rl^{Su23}/+	4.6	87

[a] Cuticular preparations were made for embryos laid by females with the indicated genotypes, and the numbers of embryos with an eighth abdominal segment (A8) were counted. Embryos with only a residual A8 were included in the counts.

Dsor1 has been demonstrated to act downstream of D-raf in the Tor pathway, and the gain-of-function mutations of *Dsor1* suppress the posterior defects in embryos devoid of terminal class gene maternal activities (Tsuda *et al.* 1993; Lim *et al.* 1997; Table 1; Figure 4, D, I, and N). The loss-of-function mutations of *rl* significantly reduced the suppressor activity of $Dsor1^{Su1}$ (Table 1), demonstrating that Rl acts downstream of Dsor1 in the Tor pathway.

A temperature-sensitive gain-of-function allele of *tor*, tor^{RL3} (Klingler *et al.* 1988), was significantly enhanced by rl^{Su23} and rl^{Su14}. As shown in Table 2, considerable numbers of embryos produced by females heterozygous

TABLE 2

Enhancement of tor^{RL3} by gain-of-function mutations in *rl* and *Dsor1*

Maternal genotype	% of embryos hatched at 17°	at 28°
+/+; + tor^{RL3}/++	99.6 (553)	94.4 (648)
+/+; + tor^{RL3}/rl^{Su14} +	97.8 (538)	79.6 (1159)
+/+; + tor^{RL3}/rl^{Su23} +	80.8 (616)	12.9 (822)
$Dsor1^{Su1}$/+; + tor^{RL3}/+ +	94.8 (2111)	26.8 (6030)
$Dsor1^{Su1}$/$Dsor1^{Su1}$; + tor^{RL3}/+ +	82.9 (2785)	3.9 (2137)

Numbers in parentheses indicate the numbers of embryos counted.

TABLE 3

Proliferation defects in loss-of-function mutations of *Dsor1* and *D-raf* and their suppression by a gain-of-function mutation in *rl*

Genotype[a]	No. of twin spots analyzed	Mean no. of doubling		Relative rate (A/B)	Normalized rate
		mwh (A)	f^{36a} (B)		
+/*M2′*; *mwh*/*mwh*	24	7.28 ± 1.56	7.87 ± 1.48	0.930 ± 0.141	1.00
+/*M2′*; rl^{Su23}/+; *mwh*/*mwh*	13	7.52 ± 0.86	7.87 ± 1.38	0.974 ± 0.138	1.05
D-raf^{1}/*M2′*; *mwh*/*mwh*	24	3.37 ± 1.73	5.89 ± 0.60	0.572 ± 0.190	0.62
D-raf^{1}/*M2′*; rl^{Su23}/+; *mwh*/*mwh*	13	7.52 ± 0.86	7.87 ± 1.38	0.947 ± 0.138	1.02
Dsor1^{Gp158}/*M2′*; *mwh*/*mwh*	44	2.06 ± 1.46	6.66 ± 1.22	0.304 ± 0.227	0.33
Dsor1^{Gp158}/*M2′*; rl^{Su23}/+; *mwh*/*mwh*	14	4.48 ± 1.43	6.91 ± 1.56	0.661 ± 0.193	0.71
Dsor1^{r1}/*M2′*; *mwh*/*mwh*	13	4.82 ± 1.67	8.28 ± 1.77	0.585 ± 0.166	0.63
Dsor1^{r1}/*M2′*; rl^{Su23}/+; *mwh*/*mwh*	13	8.65 ± 0.32	8.91 ± 0.33	0.977 ± 0.031	1.05

[a] *M2′* is *Dp(1; Y; 3)M2′*, *mwh*$^{+}$ *ve*$^{+}$ *FR1*, *y cv v f*^{36a}; *mwh ve h* (Tsuda *et al.* 1993).

for *tor*^{RL3} and also for either rl^{Su23} or rl^{Su14} failed to hatch at 28°. Most of the nonviable embryos had a reduced number of abdominal segments (data not shown).

The above results demonstrate the involvement of Rl in the Tor signaling pathway, and it is likely that the gain-of-function mutations of *rl* suppress the terminal defects of the terminal class mutant embryos similar to the gain-of-function mutations of *Dsor1* (Tsuda *et al.* 1993; Lim *et al.* 1997). Contrary to expectations, rl^{Su23} and rl^{Su14} did not suppress *fs(1)ph* at all: Neither the cuticular pattern nor the expression patterns of *tll* and *hkb* in the embryos lacking maternal *fs(1)ph* were affected by rl^{Su23} or rl^{Su14} (Table 1; Figure 4, E, J, and O).

Functions of *rolled* and *Dsor1* in cell proliferation: Loss-of-function *rl* mutants die as third instar larvae that lack imaginal discs (Hilliker 1976), suggesting the involvement of *rl* in the proliferation of imaginal disc cells. Animals hemizygous for loss-of-function mutations of *D-raf* showed a similar phenotype (Perrimon *et al.* 1985; Nishida *et al.* 1988; Tsuda *et al.* 1993; Hata *et al.* 1994). Clonal analysis demonstrated that the rate of proliferation was reduced ~40% in clones homozygous for null *D-raf*^{1} (Tsuda *et al.* 1993; Hata *et al.* 1994; Table 3). A clonal analysis in the present study demonstrated that proliferation was much more severely affected in null *Dsor1*^{Gp158} than in null *D-raf*^{1} (Table 3). The rate of proliferation in *Dsor1*^{Gp158} was greatly reduced, and only one or two doubling events took place in most of the *Dsor1*^{Gp158} clones, while more than six doublings occurred in their sibling clones. Proliferation rates in the clones homozygous for the *Dsor1* hypomorphs, *Dsor1*^{r1} and *Dsor1*^{r2}, were affected in a similar manner to those observed in *D-raf*^{1} (Tsuda *et al.* 1993; Table 3).

To elucidate the function of *rl* in cell proliferation, we tested whether rl^{Su23} suppresses the proliferation defects in the *D-raf* and *Dsor1* mutant clones. As shown in Table 3, rl^{Su23} suppressed the proliferation defects in the *D-raf* and *Dsor1* mutant clones, although it did not restore the viability of the flies hemizygous for *D-raf*^{1}, *Dsor1*^{r1}, or *Dsor1*^{r2}. This indicates that Rl acts downstream of Dsor1 in the signaling pathway regulating the imaginal cell proliferation. It should also be noted that the proliferation defects in null *Dsor1*^{Gp158} were significantly suppressed by rl^{Su23}, suggesting considerable basal level activity by rl^{Su23}.

DISCUSSION

Function of *rolled* in the Torso signaling pathway: Genetic and molecular characterization of dominant suppressors of *D-raf* on the second chromosome identified two gain-of-function alleles of *rl* with different dominant activities: rl^{Su14} (weak) and rl^{Su23} (strong). Surprisingly, the molecular lesion associated with rl^{Su23} was found to be identical to that of rl^{Sem} (Brunner *et al.* 1994). In fact, the dominant activity of rl^{Su23} on the eye and wing morphology was similar to that observed in rl^{Sem}. It has been reported that rl^{Sem} is invariably dominant female sterile with a dominant *tor*-like phenotype (Brunner *et al.* 1994), whereas both rl^{Su23} and rl^{Sem} showed only a weak dominant female sterility in this study. This discrepancy would be due to a difference in the genetic background: either the presence of a mutation(s) that enhances Tor signaling in the original rl^{Sem} stock or the occurrence of a suppressor mutation(s) in the current stocks of both rl^{Sem} and rl^{Su23}. So far, outcrossings of the current stocks have shown no evidence of suppressor mutations.

The cell fates at the anterior and posterior termini of the early embryo are determined by the Tor signaling pathway (Nüsslein-Volhard *et al.* 1987; Lu *et al.* 1993b). Unfortunately, the significance of *rl* in the pathway could not be directly assessed with germline clones because of its proximity to the centromeric heterochromatin (Berghella and Dimitri 1996). However, a half-reduction in the gene dosage of *rl* significantly reduced the suppressor activity of the dominant *Dsor1* mutation on the posterior defect in the embryos lacking terminal

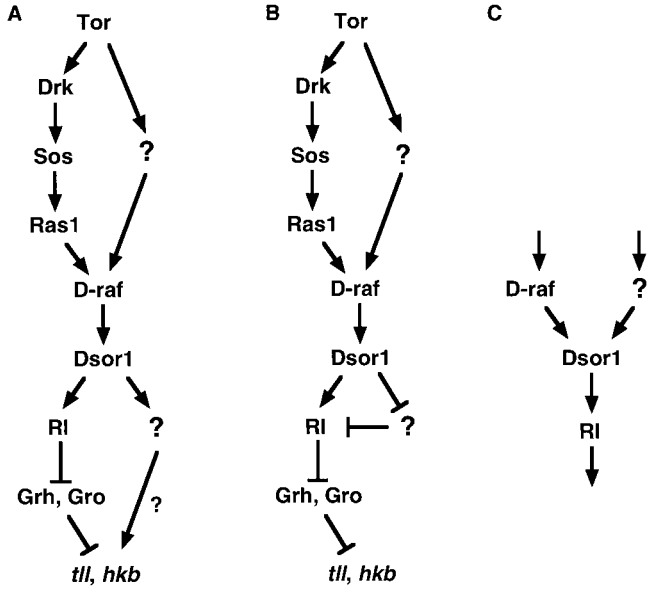

Figure 5.—Models for the Tor signaling pathway at the embryonic posterior end (A and B) and for the cascade regulating cell proliferation (C). The Tor receptor activation at the posterior end of the early embryo causes local activation of *tll* and *hkb* by antagonizing repressor activities of Grainyhead (Grh) and/or Groucho (Gro; Liaw *et al.* 1995; Paroush *et al.* 1997). It has been suggested that Ras1 mediates only a part of the signals from the Tor receptor and that D-raf integrates signals from Ras1 and another yet-unidentified factor (Hou *et al.* 1995; A and B). Loss-of-function mutations of *rl* markedly reduced the suppressing activity of $Dsor1^{Su1}$ on $fs(1)ph^{1901}$, and this clearly indicates that Rl acts downstream of Dsor1 in this pathway. However, the inability of gain-of-function mutations in *rl* to suppress the terminal class mutations (Figure 4, E, J, and O) suggests that activation of Rl is not sufficient for Tor signaling. One possible explanation would be that Rl transduces only a portion of the signals from Dsor1 and that another unknown factor participates in the signaling in addition to Rl (A). It is also possible that Dsor1 relieves Rl from inhibition by an unidentified antagonizing factor in addition to activating Rl (B). Clonal analysis demonstrated that the proliferation defect in null *Dsor1* mutant clones is much more severe than in null *D-raf* clones, and this can be explained if D-raf mediates only a part of signals and Dsor1 integrates signals from other unknown factors as well as from D-raf (C).

class gene maternal activities (Table 1). This indicates that Rl is required in Tor signaling and that it acts downstream of Dsor1 (Figure 5). A detailed analysis of the gain-of-function mutations of *rl* demonstrated that they significantly strengthened the signals from the Tor receptor tyrosine kinase. The dominant activity is rather moderate and explains the observed weak dominant sterility (Figure 4 and Table 2). It also should be noted that the dominant *rl* mutations exhibited no suppressor activity on the terminal class mutations, in contrast to the dominant *Dsor1* mutations (Figure 4; Table 1). The significance of this observation is discussed in a later section.

MAPK cascade and cell proliferation: The clonal analysis demonstrated that *D-raf* and *Dsor1* encode the essential components of the signaling pathway regulating proliferation of imaginal disc cells (Tsuda *et al.* 1993). The proliferation defects of loss-of-function mutations of *D-raf* and *Dsor1* were significantly suppressed by rl^{Su23}, indicating that Rl acts downstream of Dsor1. It should also be noted that the proliferation defect in the null $Dsor1^{Gp158}$ clones is much more severe than in the null $D\text{-}raf^{1}$ clones (Table 3). This may suggest that D-raf mediates only a portion of the signals for proliferation to Dsor1 and that Dsor1 integrates growth-stimulating signals from other unknown factor(s) as well (Figure 5C).

Signal branching and integration in the MAPK cascade: As described above, the dominant *rl* mutations exhibited no suppressor activity on the terminal class mutations. This could be explained if the *rl* gain-of-function mutations were devoid of constitutive activity, and if the expression of their dominant activity were strictly dependent on upstream signals. However, the significant suppressor activity of rl^{Su23} in the proliferation defects in the null $Dsor1^{Gp158}$ clones and in the R7 cell fate decision in the null *sev* mutants may run counter to this assumption.

It has been reported that the increased signal sensitivity of the mammalian $ERK2^{D319N}$ protein that has a mutation analogous to Rl^{Sem} is due to a decreased sensitivity to dual-specificity MAPK phosphatases such as PAC1, CL100/MKP-1, MKP-2, and MKP-3 rather than to an increased kinase activity (Bott *et al.* 1994; Chu *et al.* 1996; Camps *et al.* 1998). On the other hand, an *in vitro* kinase assay of the recombinant Rl^{Sem} mutant protein produced in bacteria demonstrated significant activity for the phosphorylation of Yan, a native substrate of Rl, in the absence of activating MAPKK, while the normal recombinant Rl did not (Oellers and Hafen 1996). In the presence of activated mammalian MAPKK, Rl^{Sem} exhibited a higher kinase activity than Rl^{+}. The latter observations suggest an increased basal level activity of Rl^{Sem} in addition to an increased sensitivity to the activator. The constitutive activity observed *in vitro* is consistent with the significant suppressor activity of rl^{Su23} in the proliferation defect in the null $Dsor1^{Gp158}$ clones (Table 3). Taking these observations into account, it is most likely that the dominant activity of the Rl^{Sem} and Rl^{Su23} mutant proteins is due to both an increased basal level activity and a decreased sensitivity to inactivating phosphatases.

On the basis of the above considerations, we propose that the activation of Rl is necessary but not sufficient for Tor signaling, and that Dsor1 may provide yet another branching point in the Tor signaling pathway. One possible model would be that Dsor1 activates another unknown factor in addition to Rl in the Tor pathway, and that both are required for the transcriptional activation of *tll* and *hkb* (Figure 5A). It would also be possible that an inactivation of a factor that antagonizes the Rl function by Dsor1 would be required for the activation

of the pathway (Figure 5B). Defects of varying degrees were seen in mitoses in the syncytial blastoderm embryos devoid of the maternal *Dsor1* activity (L. Tsuda, H.-Y. Ha and Y. Nishida, unpublished observations), suggesting that Dsor1 participates in the regulation of mitosis and is activated throughout the embryo during cleavage divisions. Bifurcation of the Tor signals downstream of Dsor1 may constitute a mechanism for preventing Dsor1 from activating the target genes in regions other than the terminal regions of the embryo. As discussed above, integration of signals for imaginal cell proliferation would then take place at some other point in the MAPK cascade (Figure 5C). The differential branching and integration of signals may contribute to the functional diversification of the ubiquitous MAPK cascade.

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