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Abstract

The rolled (rl) gene of Drosophila encodes a homologue of vertebrate mitogen-activated protein kinases. Genetic analyses have shown that the gain-of-function mutation rolledSevenmaker (rlSem) is sufficient to activate developmental pathways controlled by distinct receptor tyrosine kinases, such as Sevenless, Torso, and the Drosophila epidermal growth factor receptor homologue. Here we show that mutant RLSem protein, immunoprecipitated from transiently transfected COS cells, exhibits a moderate increase in kinase activity compared with wild-type RL protein. Time course studies revealed that RLSem is more active than RL following short term as well as prolonged treatment with epidermal growth factor. Interestingly, a more pronounced difference in kinase activity is observed when the proteins are immunoprecipitated from extracts of Drosophila rl and rlSem larvae. In fact, the kinase activity of RLSem from larvae extracts is comparable to the kinase activity of larvae expressing either an activated Sevenless receptor or an activated Raf kinase. We also demonstrate that Dsor1, which has been placed upstream of rl genetically, is able to phosphorylate and activate RL in vitro.
Biochemical Characterization of Rolled^{Sem}, an Activated Form of Drosophila Mitogen-activated Protein Kinase*

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The rolled (rl) gene of Drosophila encodes a homologue of vertebrate mitogen-activated protein kinases. Genetic analyses have shown that the gain-of-function mutation rolled^{Sevenmaker} (rl^{Sem}) is sufficient to activate developmental pathways controlled by distinct receptor tyrosine kinases, such as Sevenless, Torso, and the Drosophila epidermal growth factor receptor homologue. Here we show that mutant Rl^{Sem} protein, immunoprecipitated from transiently transfected COS cells, exhibits a large increase in kinase activity compared with wild-type Rl protein. Time course studies revealed that Rl^{Sem} is more active than Rl following short term as well as prolonged treatment with epidermal growth factor. Interestingly, a more pronounced increase in kinase activity is observed when the proteins are immunoprecipitated from extracts of Drosophila rl and rl^{Sem} larvae. In fact, the kinase activity of Rl^{Sem} from larvae extracts is comparable to the kinase activity of larvae expressing either an activated Sevenless receptor or an activated Raf kinase. We also demonstrate that Dsor1, which has been placed upstream of rl genetically, is able to phosphorylate and activate Rl in vitro.

The mitogen-activated protein kinase (MAPK) cascade is a major signaling system by which cells translate extracellular signals into intracellular responses. The mammalian extracellular signal-regulated kinases (ERKs) are the best studied members of the MAP kinase family (for reviews, see Refs. 1 and 2). They are activated by phosphorylation on threonine and tyrosine residues by dual specificity MAPK kinases (MAPKKs). On activation, MAP kinases translocate to the nucleus (3) and activate transcription factors through phosphorylation of serine or threonine residues in the motif P/LXT/SP. One of the best characterized target proteins of MAPK is the ternary complex factor ELK-1, which is phosphorylated by MAP kinases in vitro on sites essential for trans-activation by the serum response element on the c-fos promoter in vivo (4, 5).

Many steps of the MAP kinase cascade are conserved in different species, and homologous components have been identified in mammals, yeast, Drosophila melanogaster, and Caenorhabditis elegans. In Drosophila, for example, the specifica-

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; DMERK, Drosophila melanogaster ERK; MAPKK, mitogen-activated protein kinase kinase; GST, glutathione S-transferase; MBP, myelin basic protein; EGF, epidermal growth factor.

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subcloned as BamHI-EcoRI fragments into the pGEX2T vector and expressed exactly as described for the GST fusion proteins RlP2, DsorP2, PntP2\textsuperscript{151A}, and Yan-A (8). Mammalian GST-MAPKK fusion protein was provided by Sally Cowley (Chester Beatty Laboratories, London, United Kingdom) and the pGEX1-Yan-A construct was provided by Gerry Rubin (University of California, Berkeley, CA) (13).

Wild-type and mutant forms of rl were cloned as Asp\textsuperscript{154}-EcoRI fragments into the mammalian, SV40-based cell culture vector pMT21 (GenoTech Institute). To construct pMT21-rl-myc and its mutant derivatives, a polymerase chain reaction fragment was generated using a reverse primer that deleted the natural termination codon and added an additional sequence encoding the amino acids of the Myc epitope (GGEQKLISEEDD) followed by a termination codon and a XhoI recognition site.

Site-directed mutagenesis was performed to generate the constitutively activated (Dsor\textsuperscript{act}) and kinase-defective (Dsor\textsuperscript{kd}) mutants of Dsor1 (wild-type Dsor1 cDNA was provided by Y. Nishida, Aichi Cancer Center Research Institute, Nagoya, Japan). To generate Dsor1\textsuperscript{act}, the oligonucleotide 5'-GGTGCCCACAAATTCGTTGGCCATCCTGCGTACCGTTG-3' (antisense) was used to introduce AG to CT nucleotide changes at positions 1755 and 1756 of the genomic Dsor1 DNA sequence (GenBank accession number L14586). To generate Dsor1\textsuperscript{kd}, an additional sequence encoding the amino acids of the Myc epitope (GGEQKLISEEDD) was introduced at position 1762 of the genomic Dsor1 DNA sequence, using the following oligonucleotide: 5'-GGGACACGGCGAAAGCACTGCTCATTCCTTGATG-3' (antisense). In each case the presence of the mutated sequence was confirmed by sequencing.

The entire coding regions of wild-type and mutant Dsor1 cDNA fragments were amplified by polymerase chain reaction, using a primer containing a 5'-BamHI recognition sequence, and subcloned as BamHI fragments into the T7 promoter-containing pET3a expression vector (14). For protein expression, these plasmids were transformed into the Escherichia coli strain B21 (DE3) FLYS\textsuperscript{S}, and the recombinant proteins were expressed and purified as described (15, 16).


**Transient Transfections of COS-1 Cells**—COS-1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. Transfections were carried out as described previously (17). In brief, transient transfections were performed by the DEAE-dextran method, and cells were starved in serum-free DMEM 16 h before harvesting. Where indicated, cells were stimulated with EGF (10 ng/ml) for 15 min immediately prior to cell harvesting. Cells were washed twice in ice-cold Tris-buffered saline, scraped off the plate, and lysed for 10 min on ice in 20 mM Tris (pH 8), 40 mM Na\textsubscript{2}PO\textsubscript{4}, 50 mM NaCl, 0.1 mM MgCl\textsubscript{2}, 100 mM Na\textsubscript{3}VO\textsubscript{4}, 1 μM PFA, 0.5% sodium deoxycholate, 20 mM glucose, 0.5% phenylmethanesulfonyl fluoride (S buffer). Cells were centrifuged for 10 min at 4°C, and supernatants were stored at −70°C.

**Determination of MAPK Activation**—40 μg of protein from each COS-1 cell lysate was subjected to SDS-polyacrylamide gel electrophoresis and Western blotting to detect the slow-migrating, phosphorylated form of MAPK. In detail, after electrophoresis proteins were transferred to nitrocellulose, blots were blocked in 5% nonfat dried milk in TBST (10 mM Tris (pH 7.5), 150 mM NaCl, and 0.05% Tween 20) for 1 h and then probed with 9E10 monoclonal antibody (BAbCO), diluted 1:3000 in blocking buffer, for 1 h. After washing in TBST and incubating with goat anti-mouse serum coupled with horseradish peroxidase (diluted 1:15000 in TBST), for 1 h, the blots were washed extensively and developed using the ECL detection system (Amersham Corp.).

**Determination of MAPK Activity**—COS-1 cells were transfected with wild-type and mutant RL constructs, serum deprived, EGF stimulated, and lysed as described above. The lysates, containing equal amounts of MAPK protein, were incubated for 1.5 h at 4°C with rotation in the presence of monoclonal antibody 9E10 prebound to protein G Sepharose beads (Sigma). The beads were washed two times with S buffer (without phenylmethylsulfonyl fluoride), once with 30 mM Tris (pH 8), and once with kinase buffer (30 mM Tris (pH 8), 20 mM MgCl\textsubscript{2}, and 2 mM MnCl\textsubscript{2}). Each immunoprecipitate was incubated for 30 min at 30°C in kinase buffer containing 10 μM unlabeled ATP, 7.5 μM of MBP and 5 μCi of [γ\textsuperscript{32}P]ATP, and the reactions were terminated by adding SDS-polyacrylamide gel electrophoresis sample buffer. Incorporation of 32P into MBP was quantified by running the samples on 15% polyacrylamide slab gels, transferring to nitrocellulose, autoradiography, scanning into a PhosphorImager (Molecular Dynamics), and analyzing the intensity of each band using ImageQuant Software.

To analyze MAPK activity in *Drosophila* larvae extracts, 20 third instar larvae for each genotype were homogenized in S buffer and centrifuged for 10 min at 4°C. The extracts, split into two, were incubated for 1.5 h at 4°C with rotation in the presence of [γ\textsuperscript{32}P]ATP, and the reactions were terminated by adding SDS-polyacrylamide gel electrophoresis sample buffer. Incorporation of 32P into MBP was quantified by running the samples on 15% polyacrylamide slab gels, transferring to nitrocellulose, autoradiography, scanning into a PhosphorImager (Molecular Dynamics), and analyzing the intensity of each band using ImageQuant Software.

**Generation of Activated and Kinase-negative Mutant Versions of RL and Dsor1—**To study the biochemical properties of RL and Dsor1, eukaryotic and bacterial expression vectors encoding wild-type, activated, and inactive versions of these proteins were generated. A kinase-inactive version of RL\textsuperscript{sem} was generated by changing Lys\textsuperscript{867} in the putative ATP-binding site to Met (RL\textsuperscript{kd} (kinase defective)), and a kinase-inactive Dsor1 was generated by changing Asp\textsuperscript{224} of the conserved protein kinase motif Asp-Phe-Gly (19) to Ala (Dsor1\textsuperscript{kd}). To generate an active version of Dsor1, Ser\textsuperscript{254} and Ser\textsuperscript{258}, analogous to those sites that are phosphorylated to activate mammalian MAPKK, were changed to Glu (Dsor1\textsuperscript{a5E}), thus introducing a negative charge at these sites in an attempt to mimic phosphorylation (20, 21). The activated version of RL, RL\textsuperscript{sem}, was identifi ed genetically as a dominant gain-of-function mutation and was shown to result in a single amino acid substitution (Asp\textsuperscript{254} → Asn) in kinase domain XI (8). To easily distinguish and purify recombinant proteins, a sequence encoding an epitope from the e-Myc protein, recognized by the 9E10 monoclonal antibody (22), was fused to the C-3′ end of wild-type and mutant RL coding sequences. Fig. 1 shows a schematic diagram of the proteins analyzed in this study.

**Phosphorylation and Activation of RL in Mammalian Cells—**To investigate the biochemical properties of RL and RL\textsuperscript{sem}, we took advantage of the well established growth factor induction system of mammalian tissue culture and investigated phosphorylation and activation of these proteins.

We transiently transfected COS-1 cells with expression vectors encoding wild-type and mutant RL, harvested from either quiescent or EGF-stimulated cells, and the corresponding cell lysates to Western blot analysis using the 9E10 anti-Myc antibody. Wild-type, kinase-inactive (RL\textsuperscript{kd}), and activated (RL\textsuperscript{a5E}) versions of RL were phosphorylated in lysates of EGF-stimulated cells, as indicated by a band of reduced electrophoretic mobility (Fig. 2A). This suggests that RL is phosphorylated in this heterologous COS cell system in an EGF-de-
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dependent manner, presumably by endogenous MAPKK. Similar results were obtained by immunostaining with an anti-Rl polyclonal antibody (DmERKA; Ref. 18), which specifically detects Drosophila RI and not the endogenous mammalian MAPK protein (data not shown).

To measure the activity of wild-type and mutant RI kinases, COS-1 cells were transfected with the corresponding constructs and stimulated with EGF. RI proteins were immunoprecipitated from lysates of these cells, and their ability to phosphorylate myelin basic protein (MBP, a standard MAPK substrate) was determined. Immunoprecipitates from cells transfected with the empty vector or expressing the kinase-inactive mutant (RI\textsuperscript{kd}) exhibited no MBP kinase activity. In contrast, EGF-stimulated RI as well as RI\textsuperscript{Sem} showed kinase activity, measured by incorporation of radioactive phosphate into MBP (Fig. 2B). The activated RI\textsuperscript{Sem} was 2–3-fold more active than wild-type RI on EGF stimulation, a difference that was consistently observed in duplicate experiments and was not due to differences in the expression level of the two forms of RI. In the absence of EGF stimulation, RI\textsuperscript{Sem} exhibits a slightly higher intrinsic kinase activity than wild-type RI, visible only after prolonged exposure times (see below).

To elucidate potential differences in the mode of action of RI and RI\textsuperscript{Sem}, we analyzed their activity following EGF treatment over a more extensive time course (Fig. 3). The activation of RI is triphasic, with a first phase peaking at 5 min, a second phase peaking at about 30 min, and a third broad phase with a peak at 2 h followed by a slow decline to basal levels at 9 h after the initial addition of EGF. For mammalian MAPK, a similar activity was reported with a first peak at 5 min and a second peak about 3 h after EGF treatment (23, 24). In contrast, the activation of RI\textsuperscript{Sem} seems to be only monophasic. The activation reaches its peak at about 5 min and is 2–3-fold higher than RI, as we have shown before. Then the activity continuously declines to near basal levels after 9 h. Notably, throughout this time course, the activity of RI\textsuperscript{Sem} is consistently higher than that of RI.

Phosphorylation of Nuclear Target Proteins by RI and RI\textsuperscript{Sem}—In the experiments described above, we have examined the kinase activity of RI toward the artificial substrate MBP. It is possible, however, that the RI\textsuperscript{Sem} mutation affects the substrate specificity of MAP kinase. Therefore, we wanted to test whether RI and RI\textsuperscript{Sem} behave differently toward their natural substrates. We have demonstrated previously that pntP2 and yan, two genes encoding nuclear factors that, like ELK-1, belong to the ETS domain family of transcription factors, act downstream of rl in the Sev pathway. We showed that wild-type RI phosphorylates Yan and PntP2 \textit{in vitro} (11). To extend these \textit{in vitro} studies, we generated and expressed recombinant wild-type and mutant RI proteins fused to GST and performed kinase assays using bacterially expressed PntP2 and Yan-A as substrates.

RI and RI\textsuperscript{Sem} were able to phosphorylate Yan (Fig. 4) and PntP2 (data not shown) in the presence of activated mammalian MAPKK (a kind gift from Sally Cowley and Chris Marshall, Chester Beatty Laboratories), whereas kinase-inactive RI\textsuperscript{kd} showed no activity. RI\textsuperscript{Sem} exhibited 2–3-fold higher activity toward PntP2 and Yan than RI. This difference is similar to that observed in assays using MBP as a substrate (Fig. 2B). It is noteworthy here that RI\textsuperscript{Sem} exhibited weak intrinsic kinase activity toward Yan that was observed in the absence of activated MAPKK (Fig. 4).

Effects of Dsor1 on RI Activity in Vitro—So far we have shown that mammalian MAPKK is able to phosphorylate Drosophila RI kinase in COS cells. The Drosophila homologue of MAPKK, Dsor1, has been shown genetically to act in several different receptor tyrosine kinase-mediated pathways in Drosophila. By analogy to the function of MAPKK as a direct activator of MAPK in vertebrates, Dsor1 is believed to act upstream of rl. There is, however, neither genetic nor biochemical evidence demonstrating that Dsor1 directly activates RI.

**Fig. 2.** RI and RI\textsuperscript{Sem} are phosphorylated and activated in COS-1 cells. A, RI, RI\textsuperscript{Sem} and RI\textsuperscript{kd} phosphorylation was revealed as a shift in electrophoretic mobility in EGF-induced COS-1 cells, using antibody 9E10 as described under “Materials and Methods.” As a control, COS-1 cells were also transfected with the empty vector. The positions of the active (MAPK-P) and inactive MAPK are indicated. B, Western blot analysis of MAPK activation in the presence of activated MAPKK using antibody 9E10, and the MBP kinase activity of the immunoprecipitates was determined as described under “Materials and Methods.” The results were quantified with a PhosphorImager and expressed relative to the kinase activity of RI (1). Similar results were obtained in four separate experiments.

**Fig. 3.** Time course of MBP kinase activation in COS cells in response to EGF. Cells were transfected with RI (●) and RI\textsuperscript{Sem} (○), respectively, serum deprived, and stimulated with EGF for 5, 10, 20, and 30 min and 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, and 9 h. The first (1), second (2) and third (3) phases of RI activity are indicated. Results were quantified with a PhosphorImager and expressed as fold activation. The data presented are representative of five experiments, which gave similar results.
Therefore, we wanted to test whether Dsor1 is able to phospho-
ylate and activate RI in vitro. We expressed bacterial fusion
proteins of a putatively activated and kinase-defective version of
Dsor1 (see above), analogous to the mutations made in mam-
malian MAPKK (21). We carried out MBP kinase assays with
bacterially expressed Dsor1 and RI mutant versions in differ-
et combinations. Only the activated version of Dsor1,
(Dsor1<sup>act</sup>) was able to phosphorylate and activate RI and RI<sup>SEM</sup>
in vitro (Fig. 5). Wild-type Dsor1 and kinase-inactive Dsor1
(Dsor1<sup>kd</sup>) did not exhibit any kinase activity. Experiments were
also performed using PntP2 and Yan as substrates for the
kinase assays, and similar results were obtained (data not
shown). These results demonstrate that Dsor1 directly activ-
ates RI in vitro.

Activity of RI- and RI<sup>SEM</sup> Kinase in Drosophila Larvae Ex-
tracts—Surprised by the marginal differences in kinase activ-
ity of RI and RI<sup>SEM</sup> in vitro, considering the relatively strong
effects of this mutation in vivo, we decided to investigate bio-
chemically the activity of RI- and RI<sup>SEM</sup> kinase isolated from
Drosophila larvae. Protein extracts were prepared from larvae of
different genotypes and immunoprecipitated with a poly-
clonal RI-specific antibody, and their MBP kinase activity was
determined. We used wild-type (+/+) larvae containing two
copies of the rl gene, rl<sup>SEM</sup>/rl<sup>S</sup/+ larvae containing one copy of rl<sup>SEM</sup>
and a wild-type copy of rl and, also rl<sup>SEM</sup>/rl<sup>10a</sup> larvae containing
only one copy of rl<sup>SEM</sup>, since the rl<sup>10a</sup> allele represents a defi-
ciency for the entire rl locus. MAP kinase activity was detected
in all extracts (Fig. 6). RI<sup>SEM</sup>/rl<sup>S</sup> extracts showed 5–6-fold higher
activity and rl<sup>SEM</sup>/rl<sup>10a</sup> showed 8–9-fold higher stimulation
than wild-type extracts. Wild-type and mutant kinases showed
the same kinetics. The kinase assays were linear with respect
to incubation time, the amount of immunoprecipitated protein,
and the amount of added substrate protein (data not shown).

As a control, we also heat shocked larvae of the genotype
r<sup>S</sup>/r<sup>S</sup>/+, transformants carrying the Sem mutation (8), immu-
noprecipitated RI, and determined MBP kinase activity.
These extracts exhibited 4–5-fold higher kinase activity than
wild-type extracts, exactly matching the activity of rl<sup>SEM</sup>/rl<sup>S</sup> +
extracts.

These experiments demonstrate that the difference between
wild-type RI and RI<sup>SEM</sup> kinase activity in larvae is significantly
more pronounced than in heterologous systems. The higher
activity in rl<sup>SEM</sup>/rl<sup>S</sup> (only one copy of rl<sup>SEM</sup>) than in rl<sup>SEM</sup>/rl<sup>S</sup> +
(one copy of rl and one copy of rl<sup>SEM</sup>) extracts is consistent with
our genetic data. We have shown previously that the phenotype
of rl<sup>SEM</sup> is enhanced in the absence of one copy of the wild-type rl
gene (8).

To analyze the activity of endogenous RI in larvae in which
the MAP kinase pathway was activated by upstream compo-
nents, we prepared lysates from larvae expressing activated forms of
raf (raf<sup>Y9</sup>, Ref. 25) and Sev (Sev<sup>S11</sup>; 26). The larvae were
heat shocked for 1 h at 37 °C to induce ubiquitous expres-
sion of the corresponding transgenes; then RI was immunopre-
cipitated, and MBP kinase activity was determined. On heat
shock induction, RI, immunoprecipitated from raf<sup>Y9</sup> and
Sev<sup>S11</sup> extracts, was 9- and 4-fold more active, respectively,
than RI immunoprecipitated from wild-type extracts (Fig. 6).
Consistent with our genetic analysis of rl<sup>SEM</sup>, it seems that the
increase in kinase activity, generated by the Sem point muta-
tion, is comparable with that generated by constitutive activa-
tion of upstream signaling molecules.

**DISCUSSION**

The genetic characterization of the rl<sup>SEM</sup> gain-of-function
mutation demonstrated that RI plays an essential role in var-
ious developmental pathways in *Drosophila*. It did not permit
insight, however, into the mechanisms by which the rl<sup>SEM</sup>
mutation affects the biochemical properties of MAP kinase in vivo.
Here we have shown that Rl<sup>SEM</sup>, isolated from COS-1 cells or
bacteria, possesses only weakly enhanced intrinsic kinase ac-
activity toward the generic substrate MBP and its natural sub-
strates PntP2 and Yan. Both, wild-type and mutant RI proteins
are phosphorylated, and their kinase activity is stimulated by
endogenous mammalian or recombinant *Drosophila* MAPKK,
although phosphorylated Rl<sup>SEM</sup> kinase is 2–3-fold more active.
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than the wild-type Rl protein. Similar results have been reported for mammalian ERK2, containing the same mutation in the homologous position. D319N ERK2 exhibits 2-fold higher kinase activity on EGF stimulation in comparison with wild-type ERK2 (27). In Xenopus, injection of RlSem mRNA and also of ERK2mRNA (p44 MAPK carrying the corresponding D to N exchange) into embryos was sufficient to induce the expression of brachyury (Xbra), an immediate early mesoderm response gene (28, 29). Therefore, it appears that this point mutation in the kinase subdomain XI has a conserved effect on different members of the MAP kinase family.

There are a number of possibilities why RlSem is more active on stimulation than the wild-type protein. The D334N mutation in RlSem could affect the subcellular localization of the protein and thus its accessibility to activating or inactivating signals. However, in immunofluorescence studies we did not observe a difference in the subcellular localization of wild-type and mutant proteins in Drosophila imaginal discs2 or when expressed in COS-1 cells (data not shown). Alternatively, the enhanced kinase activity of RlSem could be due to an increased affinity toward its physiological substrates. However, the difference in activity of RlSem kinase compared with wild-type Rl was similar for both its natural substrates, PntP2 and Yan, and for the artificial substrate MBP. This implies that RlSem does not possess a noticeably altered substrate specificity. Another explanation for the increased activity is that RlSem is inactivated at a reduced rate, resulting in more persistent activity. It has been shown that mammalian ERK2, carrying the Sem mutation (D319N ERK2), is more resistant to the action of the MAP kinase-specific phosphatase CL100 than wild-type MAPK (27). The results of our time course experiments also support this hypothesis. We showed that the activation of Rl is triphasic in response to EGF stimulation in COS cells. A rapid activation occurring at 5 min, a short decline at 20 min, and a second activation at 30 min with a minimum at 1 h is followed by a broader wave with a peak at about 2 h after stimulation. A similar, but only biphasic, activation was already reported for mammalian MAPK in CCL39 cells, a hamster fibroblast cell line, showing a fast activation at 5 min and a broader phase at about 3 h after EGF stimulation (23, 24). In contrast, the activation of RlSem seems to be only monophasic. It peaks at about 5 min and then continuously declines to near basal levels. Throughout this time course the activity of RlSem is consistently higher than that of Rl.

It is interesting to note that the duration of MAP kinase activation has been postulated as the determinative factor in the decision between differentiation and proliferation in PC12 pheochromocytoma cells. Stimulation of PC12 cells with EGF results in a transient activation of MAP kinase and induces proliferation, whereas NGF stimulation, also mediated by the Ras-Raf pathway, results in a persistent activation of MAP kinase and induces neurite outgrowth (30; for review, see Ref. 31). In mutant rTs flies, the phenotypes observed are related to changes in differentiation, such as the excess recruitment of R7 cells, differentiation of additional wing veins, and suppression of the differentiation of the segmented trunk region of the embryo. RlSem does not affect the proliferation of cells, although rlt is clearly required for cell proliferation (32). The more persistent activity of RlSem may preferentially trigger differentiation as opposed to proliferation.

In Drosophila larval extracts the kinase activity of RlSem in comparison with wild-type Rl is considerably higher than observed in EGF-stimulated COS-1 cells or when isolated from bacteria. In fact, the level of activity was comparable to that observed in EGF-stimulated COS cells. A rapid activation occurring at 5 min, a short decline at 20 min, and a second activation at 30 min with a minimum at 1 h is followed by a broader wave with a peak at about 2 h after stimulation. A similar, but only biphasic, activation was already reported for mammalian MAPK in CCL39 cells, a hamster fibroblast cell line, showing a fast activation at 5 min and a broader phase at about 3 h after EGF stimulation (23, 24). In contrast, the activation of RlSem seems to be only monophasic. It peaks at about 5 min and then continuously declines to near basal levels. Throughout this time course the activity of RlSem is consistently higher than that of Rl.

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In Drosophila larval extracts the kinase activity of RlSem in comparison with wild-type Rl is considerably higher than observed in EGF-stimulated COS-1 cells or when isolated from bacteria. In fact, the level of activity was comparable to that observed in protein extracts of larvae expressing an activated Sev or Raf kinase. The activity was highest in rTs larval extracts (8–9-fold), in which the wild-type rlt allele is missing, corroborating genetic data showing that the phenotype of rTs is enhanced in the absence of the wild-type copy of the rlt gene (8). The question arises why in Drosophila extracts the difference in kinase activity of Rl and RlSem is higher than in heterologous systems. It is possible that the resistance of RlSem to inactivation by phosphatases is more pronounced in larval extracts than in COS cells. In addition, genetic experiments suggest that the activity of RlSem is dependent on components acting upstream in the pathway. Sos and Ras1 act as dominant

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2 D. Brunner, personal communication.
suppressor(s) of the rlSem phenotype. In this respect the behavior of rlSem differs from other gain-of-function mutations in the Sev pathway. For instance, the recruitment of additional R7 cells in flies carrying a gain-of-function mutation in sev is not suppressed by mutations in boss, which codes for the Sev ligand. The dependence of rlSem on upstream components suggests that some sort of feedback loop is important in maintaining rlSem activation. Here we have shown biochemically that rlSem can respond to activating signals with increased activity, which is consistent with the hypothesis of a positive feedback loop. Furthermore, this feedback loop may explain how, even in the absence of an inducing signal, the basal activity of the signaling cascade is sufficient to activate rlSem above a threshold required for eliciting certain cellular responses, such as R7 differentiation in the eye, vein formation in the wing, and suppression of segmentation in the embryo. Therefore, the slight increase in kinase activity may be sufficient to trigger this feedback loop and the activation of various differentiation pathways.

A task for the future is to prove the existence of such a feedback loop in vivo. The genetic characterization of second site mutations that suppress or enhance the dominant phenotype of the rlSem mutation should lead to the identification of further components acting in concert with rlSem in the signal transduction pathway.

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3 D. Brunner and E. Hafen, manuscript in preparation.

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