Biochemical Characterization of Rolled\textsuperscript{Sem}, an Activated Form of \textit{Drosophila} Mitogen-activated Protein Kinase*

Nadja Oellers\textsuperscript{1} and Ernst Hafen\textsuperscript{§}

From the Zoologisches Institut, Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

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

The mitogen-activated protein kinase (MAPK)\textsuperscript{1} 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 promotor 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, \textit{Drosophila melanogaster}, and \textit{Caenorhabditis elegans}. In \textit{Drosophila}, for example, the specification of the R7 photoreceptor cell fate in each ommatidium of the developing eye is dependent on the components of the MAP kinase cascade. In the R7 precursor cells the Sevenless (Sev) receptor tyrosine kinase is activated by its ligand, the Bride-of-sevenless (Boss) protein. The signal is then transduced via Drk (an SH3-SH2-SH3 adaptor protein, homologous to Grb2), Sos, Ras1, and the protein kinases Raf, Dsor1 (MAPKK), and \textit{Rl} (MAPK) to the nucleus (reviewed in Refs. 6 and 7). A gain-of-function mutation in the \textit{rl} gene, \textit{rl\textsuperscript{Sem}}, was identified in a genetic screen for dominant mutations that result in the specification of R7 cells in the absence of the inducing signal, the Boss ligand. This dominant mutation is caused by a single amino acid substitution (D334N) in the catalytic domain of the kinase (8). Interestingly, this mutation not only activates the \textit{Sev} pathway in the developing eye but also induces the formation of additional wing veins and causes male sterility mimicking the activation of the \textit{Drosophila} EGF receptor homologue (DER) and Torso receptor tyrosine kinases, respectively. Therefore, \textit{Rl} function is not only necessary but also sufficient to activate multiple receptor tyrosine kinase pathways in \textit{Drosophila}. The homologue of the MAP kinase activating kinase (MAPKK) encoded by the \textit{Dsor1} gene is also required for multiple developmental pathways (9). Using a temperature-sensitive mutation in \textit{Dsor1}, Hsu and Perrimon (10) showed that \textit{Dsor1} operates like \textit{Rl} in the pathways controlled by Torso, DER, and Sev.

Two nuclear target genes have been shown genetically to act downstream of \textit{Rl} in the \textit{Sev} pathway, \textit{pointed P2} (\textit{ptnP2}) and \textit{yan}, encoding two members of the ETS family of transcription factors (11, 12). Furthermore, it has been shown that wild-type \textit{Rl} directly phosphorylates \textit{Yan} and \textit{PntP2} in \textit{vitro} (11).

The ordering of the individual components in the \textit{Sev} pathway has been determined largely by constructing double mutants of different members of the cascade to examine epistatic relationships between the different gene products. Although this type of genetic analysis allows ordering of individual components by functional criteria, it does not imply direct physical interactions, nor does it reveal the biochemical mechanisms by which the signals are transduced. To investigate the biochemical properties of the first identified gain-of-function mutation in a MAP kinase gene, \textit{Rl\textsuperscript{Sem}}, we examined the kinase activity of the corresponding mutant protein, immunoprecipitated from transfected COS cell lysates and \textit{Drosophila} larval extracts. We demonstrate that \textit{Rl\textsuperscript{Sem}}, expressed in mammalian COS cells or purified from bacteria, is 2–3-fold more active than wild-type \textit{Rl}. However, when isolated from \textit{Drosophila} larvae, \textit{Rl\textsuperscript{Sem}} exhibited a 5–9-fold higher kinase activity than \textit{Rl}. In addition we present evidence that \textit{Dsor1} is a direct activator of \textit{Rl} in \textit{vitro}.

MATERIALS AND METHODS

Construction of Recombinant Expression Plasmids and Protein Expression—The entire coding region of \textit{rl} and \textit{rl\textsuperscript{Sem}} (corresponding to \textit{rlPNT386/123340} in Ref. 8) were amplified by polymerase chain reaction,

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\textsuperscript{1} The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; DmERKA, kinase; GST, glutathione S-transferase; MBP, myelin basic protein; EGF, epidermal growth factor.

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\textsuperscript{3} Supported by the Swiss National Science Foundation. To whom correspondence should be addressed. Tel.: 41-1-257-4871; Fax: 41-1-361-3185.

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Biochemical Characterization of Drosophila RlSem/MAP Kinase

subcloned as BamHI-EcoRI fragments into the pGEX2T vector and expressed as described for the GST fusion proteins Rl, PntP2, PnTp2(15A) 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(+) EcoRI fragments into the mammalian, SV40-based cell culture vector pMT21 (Genentech 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 (GGGQKLISEEDL) followed by a termination codon and a XhoI recognition site.

Site-directed mutagenesis was performed to generate the constitutively activated (Dso1m) and kinase-defective (Dso1kd) mutants of Dso1 (wild-type Dso1 cDNA was provided by Y. Nishida, Aichi Cancer Center Research Institute, Nagoya, Japan). To generate Dso1m, the oligonucleotide 5'-GGGTCCCACAATGCGTTGGCCATCTCGCGA-TCAGTTG-3' (antisense) was used to introduce AG to CT nucleotide changes at positions 1755 and 1756 of the genomic Dso1 DNA sequence (GenBank accession number L13782), altering Asp to Glu, and to introduce AGG to CTT nucleotide changes at positions 1767–1769, altering Ser238 to a Glu codon. The Asp234 codon was mutated to an Ala codon to generate the Dso1(Ala) mutant by introducing a single tG to G nucleotide change at position 1726 of the genomic Dso1 DNA sequence, using the following oligonucleotide: 5'-GGAGACGCCGAAAGCACAGATCTTGAT-3' (antisense). In each case the presence of the mutated sequence was confirmed by sequencing.

The entire coding regions of wild-type and mutant Dso1 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 pT7a expression vector (14). For protein expression, these plasmids were transformed into the Escherichia coli strain B21 (DE3) FlvS, 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 10 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 Na4P2O7, 50 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 20 mM MgCl2, 100 µM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 20 µg/ml aprotinin, and 3 µM phenylmethylsulfonyl 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 incubation 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 RI 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 MgCl2, and 2 mM MnCl2). 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-32P]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 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 RI-specific polyclonal DmERKA antibody (18), precoted to protein A-Sepharose beads (Sigma). Kinase assays on the immunoprecipitates were performed as described above. Where indicated, heat shocks were applied for 1 h at 37 °C, followed by 2 h of recovering at 25 °C prior to homogenization.

Kinase assays with bacterially expressed recombinant RI, Yan-A, and mammalian MAPKK proteins were carried out as described previously (11).

**RESULTS**

**Generation of Activated and Kinase-negative Mutant Versions of RI and Dso1**—To study the biochemical properties of RI and Dso1, eukaryotic and bacterial expression vectors encoding wild-type, activated, and inactive versions of these proteins were generated. A kinase-inactive version of RIsem was generated by changing Lys867 in the putative ATP-binding site to Met (RIkd (kinase defective)), and a kinase-active Dso1 was generated by changing Asp224 of the conserved protein kinase motif Asp-Phe-Gly (19) to Ala (Dso1kd).

To generate an active version of Dso1, Ser234 and Ser238, analogous to those sites that are phosphorylated to mammalian MAPKK, were changed to Glu (Dso1m), thus introducing a negative charge at these sites in an attempt to mimic phosphorylation (20, 21). The activated version of RI, RIsem, was identified genetically as a dominant gain-of-function mutation and was shown to result in a single amino acid substitution (Asp234 → 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-end of wild-type and mutant RI coding sequences. Fig. 1 shows a schematic diagram of the proteins analyzed in this study.

**Phosphorylation and Activation of RI in Mammalian Cells**—To investigate the biochemical properties of RI and RIsem, 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 RI, harvested from either quiescent or EGF-stimulated cells, and subjected the corresponding cell lysates to Western blot analysis using the 9E10 anti-Myc antibody. Wild-type, kinase-inactive (RIkd), and activated (RIsem) versions of RI were phosphorylated in lysates of EGF-stimulated cells, as indicated by a band of reduced electrophoretic mobility (Fig. 2A). This suggests that RI is phosphorylated in this heterologous COS cell system in an EGF-de-

![Fig. 1. Schematic drawing of wild-type and mutant RI and Dso1 proteins. Roman numerals, kinase subdomains (I–XI) according to EGF-stimulus et al. (19). The positions of the point mutations introduced by in vitro mutagenesis, are indicated above the boxes. Stippled boxes, positions of the Myc epitope.](image-url)
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 MAPK 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 in vitro (11). To extend these 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 Rl\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.
Therefore, we wanted to test whether Dsor1 is able to phosphorylate and activate Rl 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 mammalian MAPKK (21). We carried out MBP kinase assays with bacterially expressed Dsor1 and Rl mutant versions in different combinations. Only the activated version of Dsor1, (Dsor1<sup>act</sup>), was able to phosphorylate and activate Rl and Rl<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 activates Rl in vitro.

Activity of Rl- and Rl<sup>sem</sup> Kinase in Drosophila Larvae Extracts—Surprised by the marginal differences in kinase activity of Rl and Rl<sup>sem</sup>, in vitro, considering the relatively strong effects of this mutation in vivo, we decided to investigate biochemically the activity of Rl- and Rl<sup>sem</sup> kinase isolated from Drosophila larvae. Protein extracts were prepared from larvae of different genotypes and immunoprecipitated with a polyclonal Rl-specific antibody, and their MBP kinase activity was determined. We used wild-type (+/+), Dsor1<sup>act/+</sup>, Dsor1<sup>act</sup>/+, and Rl<sup>sem</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 deficiency for the entire Rl locus. MAP kinase activity was detected in all extracts (Fig. 6). Rl<sup>sem</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>Hello</sup>Y9<sup>+</sup>, transformants carrying the Sem mutation (8), immunoprecipitated Rl, and determined MBP kinase activity. These extracts exhibited 4–5-fold higher kinase activity than wild-type extracts, exactly matching the activity of r<sup>Hello</sup>/+ extracts.

These experiments demonstrate that the difference between wild-type Rl and Rl<sup>sem</sup> kinase activity in larvae is significantly more pronounced than in heterologous systems. The higher activity in r<sup>Hello</sup>/r<sup>10a</sup> (only one copy of r<sup>Hello</sup>) than in r<sup>Hello</sup>/+ (one copy of Rl and one copy of r<sup>Hello</sup>) is consistent with our genetic data. We have shown previously that the phenotype of Rl<sup>Hello</sup> is enhanced in the absence of one copy of the wild-type Rl gene (8).

To analyze the activity of endogenous Rl in larvae in which the MAP kinase pathway was activated by upstream components, we prepared lysates from larvae expressing activated forms of raf (raf<sup>Y9</sup>; Ref. 25) and Sec (Sec<sup>N11</sup>; 26). The larvae were heat shocked for 1 h at 37 °C to induce ubiquitous expression of the corresponding transgenes; then Rl was immunoprecipitated, and MBP kinase activity was determined. On heat shock induction, Rl, immunoprecipitated from raf<sup>Y9</sup> and Sec<sup>N11</sup> extracts, was 9- and 4-fold more active, respectively, than Rl immunoprecipitated from wild-type extracts (Fig. 6). Consistent with our genetic analysis of r<sup>Hello</sup>, it seems that the increase in kinase activity, generated by the Sem point mutation, is comparable with that generated by constitutive activation of upstream signaling molecules.

**DISCUSSION**

The genetic characterization of the r<sup>Hello</sup> gain-of-function mutation demonstrated that Rl plays an essential role in various developmental pathways in Drosophila. It did not permit insight, however, into the mechanisms by which the r<sup>Hello</sup> mutation affects the biochemical properties of MAP kinase in vivo. Here we have shown that Rl<sup>Hello</sup>, isolated from COS-1 cells or bacteria, possesses only weakly enhanced intrinsic kinase activity toward the generic substrate MBP and its natural substrates PntP2 and Yan. Both, wild-type and mutant Rl proteins are phosphorylated, and their kinase activity is stimulated by endogenous mammalian or recombinant Drosophila MAPKK, although phosphorylated Rl<sup>Hello</sup> kinase is 2–3-fold more active...
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 ERK5 Sem mRNA (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 promote this hypothesis. We showed that the activation of RI 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 RI.

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 r1Sem 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 r1 is clearly required for cell proliferation (32). The more persistent activity of r1Sem may preferentially trigger differentiation as opposed to proliferation.

In Drosophila larval extracts the kinase activity of RlSem in comparison with wild-type RI 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 RI.

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2 D. Brunner, personal communication.
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REFERENCES

1. Marshall, C. J. (1994) Curr. Opin. Genet. & Dev. 4, 82–89
2. Nishida, E., and Gotoh, Y. (1993) Trends Biochem. Sci. 18, 128–131
3. Chen, R. H., Sarnecki, C., and Blenis, J. (1992) Mol. Cell. Biol. 12, 915–927
4. Marais, R., Wynne, J., and Treisman, R. (1993) Cell 73, 381–393
5. Janknecht, R., Ernst, W. H., Pengoud, V., and Nordheim, A. (1993) EMBO J. 12, 5997–6004
6. Hafen, E., Dickson, B., Raabe, T., Brunner, D., Oellers, N., and van der Straten, A. (1993) Development (Suppl.) 41–46
7. Zipursky, L. S., and Rubin, G. M. (1994) Annu. Rev. Neurosci. 17, 373–397
8. Brunner, D., Oellers, N., Szabad, J., Biggs, W. H., III, Zipursky, S. L., and Hafen, E. (1994) Cell 76, 875–888
9. Tsuda, L., Inoue, Y. H., Yoo, M. A., Mizuno, M., Hata, M., Lim, Y. M., Adachi-Yamada, T., Ryo, H., Massamune, Y., and Nishida, Y. (1993) Cell 72, 407–414
10. Hsu, J. C., and Perrimon, N. (1994) Genes Dev. 8, 2176–2187
11. Brunner, D., Dicker, K., Oellers, N., Hafen, E., Scholz, H., and Kla¨mbt, C. (1994) Nature 370, 386–389
12. O’Neill, E. M., Rebay, I., Tjian, R., and Rubin, G. M. (1994) Cell 78, 137–147
13. Lai, Z.-C., and Rubin, G. M. (1992) Cell 70, 609–620
14. Studier, F. W., and Moffat, B. A. (1986) J. Mol. Biol. 189, 113–130
15. Oellers, N., Dehio, M., and Knust, E. (1994) Mol. & Gen. Genet. 244, 465–473
16. Kerkhoff, E., and Bister, K. (1991) Oncogene 6, 93–102
17. Howe, L. R., Reverso, J. G., Gomez, N., Nakielny, S., Cohen, P., and Marshall, C. J. (1992) Cell 71, 335–342
18. Biggs, W. H., and Zipursky, S. L. (1992) Proc. Natl Acad. Sci. U. S. A. 89, 6285–6289
19. Hanke, S. K., Quinn, A. M., and Hunter, T. (1988) Science 241, 42–52
20. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) Cell 77, 841–852
21. Alessi, D. R., Saito, Y., Campbell, D. G., Cohen, P., Sitanandam, G., Rapp, U., Ashworth, A., Marshall, C. J., and Cowley, S. (1994) EMBO J. 13, 1610–1619
22. Evan, G. I., Lewis, G. K., Ramsey, G., and Bishop, J. M. (1985) Mol. Cell. Biol. 5, 3610–3616
23. Meleche, S., Sewten, K., Pages, G., and Pouyssegur, J. (1992) Mol. Endocrinol. 6, 845–854
24. Lenormand, P., Sardet, C., Pages, G., L’Allemain, G., Brunet, A., and Pouyssegur, J. (1995) J. Cell Biol. 129, 1079–1088
25. Dickson, B., Sprenger, F., Morrison, D., and Hafen, E. (1992) Nature 360, 600–603
26. Basler, K., Christen, B., and Hafen, E. (1991) Cell 64, 1069–1081
27. Bott, C. M., Thorneycroft, S. G., and Marshall, C. J. (1994) FEBS Lett. 352, 201–205
28. LaBonne, C., Burke, B., and Whitman, M. (1995) Development 121, 1475–1486
29. Traverse, S., Gomez, N., Paterson, H., Marshall, C. J., and Cohen, P. (1992) Biochem. J. 288, 351–355
30. Marshall, C. J. (1995) Cell 80, 179–185
31. Diaz-Benjumea, F. J., and Hafen, E. (1994) Development 120, 569–578

suppressors 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.

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